VOLUME 10 November 1961 NUMBER 6

THE AMERICAN JOURNAL OF Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

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Correspondence concerning business matters, subscriptions and orders for reprints should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts and books for review should be sent to Dr. Paul C. Beaver, 1430 Tulane Avenue, New Orleans 12, Louisiana.

The subscription price is $10.00 per year in U.S.A.; $10.50 in Canada; $11.25 elsewhere. Single copies, when available, $2.50 in U.S.A.; $2.75 elsewhere.

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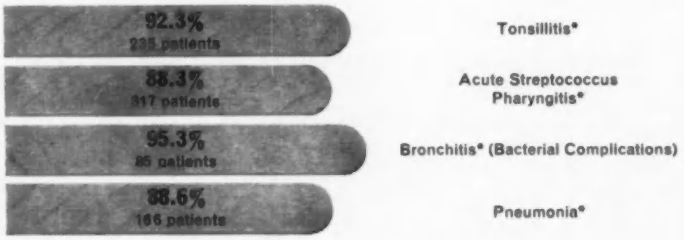

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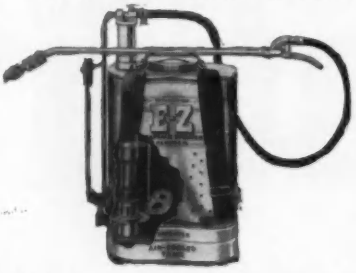

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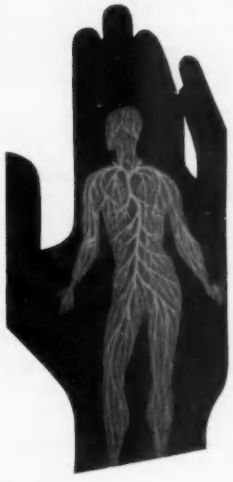

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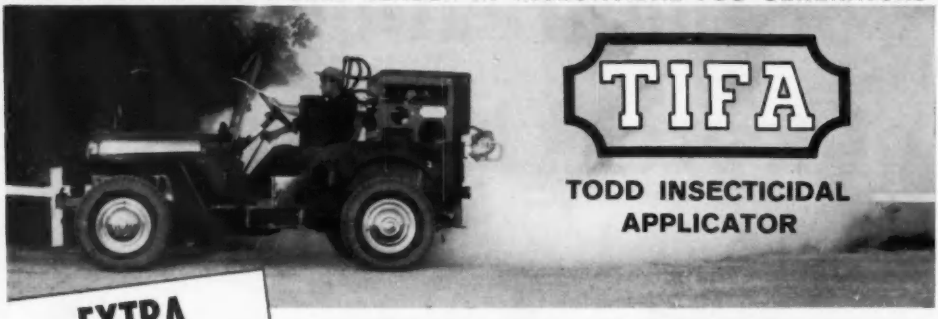

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THE 1958 MALARIA EPIDEMIC IN ETHIOPIA

RUSSELL E. FONTAINE,* ABDALLAH E. NAJJAR AND JULIUS S. PRINCE

International Cooperation Administration, Washington, D. C.

During the last half of 1958, a malaria epidemic of unusual intensity flared throughout most of the extensive highland area of Ethiopia. At least four of the central highland provinces—Shoa, Gojjam, Beghemder, and Wollo—were severely affected with numerous local districts in these provinces experiencing fulminating outbreaks accompanied by high mortality. Morbidity exceeding 75% of the population was encountered in many rural settlements. However, in the lowland provinces of Ethiopia bordering on the Sudan, Kenya, and Somalia there was no evidence of epidemics. Also, Eritrea, a federated state of north and northeastern Ethiopia, apparently escaped the epidemic as no reports of unusual malaria occurrence were issued by the Eritrean Medical Directorate.

Information on the prevalence of malaria in Ethiopia before 1958 was drawn largely from reconnaissance-type surveys. There were no sustained observations covering a span of years over a wide area of the country, and the reporting of malaria was not required and never systematically maintained. As a result, records of the occurrence of epidemics have not been documented, except vaguely in the memories of local people and in the records of a few hospitals and clinics. The important contributions to the knowledge of the epidemiology of malaria were made by Italian and British investigators, notably Covell,[1] Melville, *et al.*,[2] and Corradetti.[3, 4] These authors indicated that malaria incidence is predominantly seasonal in the greater part of the country, being associated with the heavy rains of the southeast monsoons which begin in June and end in late September. They further indicated that above 1,600 to 1,700 meters altitude the brevity of the transmission season precludes the development of effective immunity among the people, so that such areas are subject to severe periodic epidemics. Despite the general consensus that the highlands of Ethiopia are subject to periodic epidemics of malaria, there is only one unpublished report by Chabaud[5] of a severe epidemic in 1953 near Gondar, Lake Tana, in which 7,000 deaths were said to have occurred from malaria. It should be noted, however, that all gradations of malaria incidence exist in Ethiopia, varying between the extremes of very high stability along the courses of permanent rivers in the lowlands and the extremely unstable malaria which characterizes the incidence in the cooler highlands. Although malaria is widely distributed, the areas of transmission are often discontinuous, being isolated by geographical barriers such as deserts and mountains.

The variable forms of malaria incidence result from the variable climate of the country which in turn is influenced by the highly variable topography. The outstanding land feature of Ethiopia is the extensive plateau and mountain terrain which comprises most of the central and northern area of the country. Surrounding the highlands are extensive, sparsely inhabited, savannas, semi-arid plains, and deserts, parts of which lie below sea level. The configuration of the highlands is very irregular, and a variety of land forms can be found. There are mountain ranges rising well above 4,000 meters, rolling grassy plains, broad fertile valleys, and deep canyons formed by the major rivers and their tributaries. Most of the country lies above 1,000 meters elevation, and the heaviest concentration of its estimated 18,000,000 inhabitants lives above this elevation. As shown on the accompanying map, Ethiopia is situated between 4° and 16° north latitude in East Africa and, with the federated state of Eritrea, covers an area of nearly 400,000 square miles (Figure 1).

While the lowlands are largely hot and dry, there is a striking cooling and freshening of the climate as one rises to the central highlands. Above 1,500 meters elevation, nights are cool and the days agreeably warm. At Addis Ababa (the capital of Ethiopia), which lies between 2,300 and 2,500 meters elevation, the mean annual temperature ranges between a minimum of 15°C and a maximum of 18°C. The mean annual rainfall exceeds 1,200 mm. During De-

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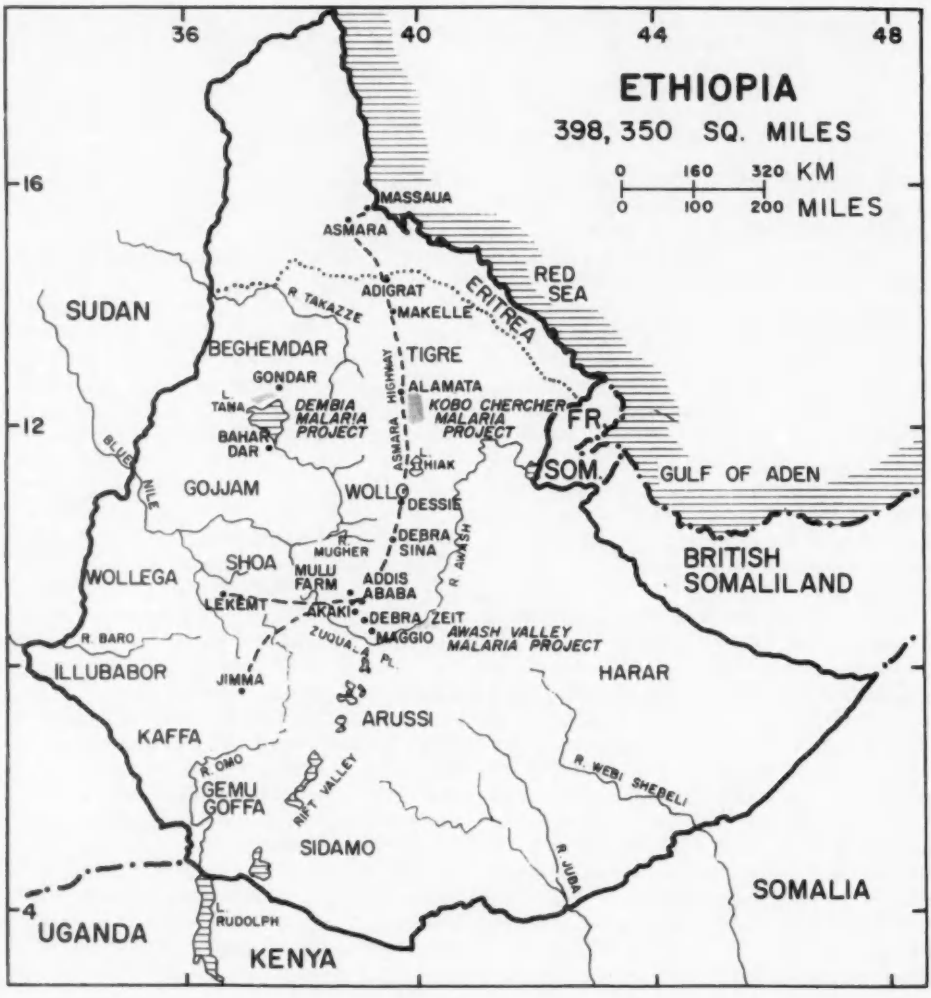

FIGURE 1

cember and January, minimum temperatures of 1°C are frequently recorded. At the elevation of Addis Ababa, it is doubtful that temperatures are maintained at a level of sufficient warmth to permit malaria transmission except in rare cases. However, not far below the city, roughly at the 2,100 meters elevation, the zone of seasonal and cyclic epidemic malaria begins and continues down to about 1,600 meters elevation.

It has been established by the authors previously referred to that the main malaria vector is *Anopheles gambiae*, and that *Plasmodium falciparum* is the predominant malaria parasite. To *A. gambiae* is attributed the responsibility for periodic, cyclic epidemics as well as recurrent seasonal outbreaks. *Anopheles funestus* is the most important of a number of secondary vectors. It appears to be mainly responsible for the maintenance of endemic malaria in the proximity of rivers, marshes, and other permanent mosquito-breeding places.

The dominance of *P. falciparum* in the distri-

bution of plasmodia species is indicated in the following series of 3,000 blood films taken by the International Cooperation Administration (ICA) malaria teams from representative localities throughout Ethiopia at different seasons of the year: *P. falciparum* 60%, *Plasmodium vivax* 25%, and *Plasmodium malariae* 15%. As far as is known, *Plasmodium ovale* has not been identified in Ethiopia.

The account of the epidemic which follows was drawn from personal observations and investigations by the authors and reports made by anti-epidemic workers who participated in an anti-epidemic campaign.

General Observations of the 1958 Epidemic

In retrospect it appears that the malaria epidemic of 1958 began in late June of 1958, although no one in the medical services at the time knew or suspected that an epidemic of unusual proportions was in its beginning stages.

By coincidence, the ICA malaria survey and control team discovered a malaria outbreak of unusual severity while conducting routine investigations in late June, 1958. The outbreak was noted near the area of Bahar Dar, where the Blue Nile River emerges from Lake Tana at an elevation of 1,800 meters. In this district seasonal outbreaks of malaria, usually of short duration and mild intensity, may appear in the months of October and November. While it has been reported by Covell[6] that slight outbreaks may occur in June at the beginning of the wet season, the findings of the ICA team suggested widespread illness of uncommon severity. The only record of malaria prevalence in this area is a spleen rate of 14% among 114 school children examined by Covell (at Bahar Dar) in October, 1952. The people living in the district were greatly distressed over the prevalence of illness, as June is not generally known to be an unhealthy month.

In June thick film blood examinations of 35 suspected malaria cases at Ganje, a village on the Adessa River near Bahar Dar, yielded 22 positive for *P. falciparum*, 1 *P. vivax*, and 3 mixed infections. Blood examinations of 48 residents of Sebatamit, a village located a few kilometers outside Bahar Dar, revealed 10 positives, 9 of which were *P. falciparum*, and 1 mixed infection. In Bahar Dar there were 9 positive *P. falciparum* and 1 *P. vivax* out of 137 blood film examinations of school children and occupants of the local jail. The combined gametocyte rate for all infections at Ganje, Sebatamit, and Bahar Dar was 31%.

A. gambiae was the only vector found in aspirator collections in human dwellings. At Ganje 100 *engorged females* was the average catch from five dwellings in a collection time of 15 minutes per dwelling.

Later in the season, the whole of the Lake Tana region of Beghemder Province and the upper Blue Nile valley experienced a catastrophic epidemic which prostrated the majority of the population in most districts. The only malaria free district in the large territory was the DDT residual sprayed Dembia Plain, ICA-assisted malaria pilot project covering an area of 2,500 square kilometers on the north end of Lake Tana. Only 80 cases were reported from the project with an estimated population of over 60,000, and there were *no* deaths attributed to malaria. A survey made in October, 1956, at about the peak of the malaria season before residual spraying, yielded 12.3% positive in the project area out of 749 blood examinations in an estimated population of 60,000. A survey in June of 1957, at the low point of seasonal incidence, yielded 3.0% positive in 293 examinations. The project, which was in the area of the 1953 epidemic noted above, was initiated in 1957 when all dwellings were residual-sprayed with DDT at the rate of 2 g of DDT per m^2 of wall space.

The epidemic which reached such an intensity in the Lake Tana region was duplicated in general throughout most other highland provinces including Shoa, Gojjam, Wollo, and portions of Wollega, Arussi, Harar, and Sidamo. A few districts in Tigre Province also reported epidemics.

In the early stages of the epidemic, public health officials sometimes assumed that the outbreaks being reported were epidemic typhus or epidemic relapsing fever because of the relatively high altitude of the affected areas. In some instances emergency anti-epidemic measures were instituted against these diseases before it was discovered through laboratory diagnosis that malaria was the cause of the outbreaks.

In Shoa Province reports of epidemics were received from numerous districts. However, relatively few could be visited by qualified investigators and then only briefly. The most complete and reliable information on the progress of the epidemic was gathered from the Zuquala Plain (1,850 meters elevation) and the Lake Akaki district (2,000 meters elevation) situated approxi-

mately 20 km south of Addis Ababa. The first indication of an epidemic in that district came to our attention in early July, 1958, through a request for assistance received from a large landholder who became alarmed over the large number of his workers falling ill with a disease which he thought might be malaria. Malaria was confirmed by laboratory diagnosis, and it was noted further that most infections were caused by *P. falciparum*. The epidemic in Zuquala and Akaki continued well into December, 1958, at which time malaria was still a common illness in those settlements and villages whose inhabitants had not been treated with chloroquine tablets distributed by anti-epidemic workers. Where possible, treatment of individuals with fever in the Zuquala area generally consisted of the administration of an immediate single dose of four 150 mg base tablets followed by two tablets daily for 3 days. Apparently only a minority of the population of Zuquala and Akaki, which is estimated at 150,000 inhabitants, escaped malaria. In both districts *A. gambiae* was the only vector found in collections made from human dwellings during July through December.

Although local residents speak of malaria epidemics in the Akaki-Zuquala area, they indicated that no epidemic of equal intensity and scope had occurred in at least 15 years.

The towns of Akaki, Debra Zeit, and Moggio, situated on the main highway passing through the Zuquala Plain, had been residual sprayed with DDT in June, 1958, by the Awash Valley Malaria Pilot Project of the World Health Organization (WHO). It was assumed that the spray gave good protection, as no evidence of an epidemic was noted in these places.

From Zuquala the epidemic extended many kilometers up the headwaters of the Awash River, where severe outbreaks were observed as high as 2,150 meters. A number of locally transmitted outbreaks were disclosed in villages at 2,000 and 2,150 meters elevation on the highway between Addis Ababa and Jimma, only 35 km from Addis Ababa.

Northwest of Addis Ababa severe malaria outbreaks were reported early in August from the Mugher River valley and other tributary river valleys of the Blue Nile. The topography of the area is characterized by elevated tableland 2,200 to 2,800 meters high, interspersed by canyons and valleys 300 to 800 meters lower than the surrounding tableland. This land configuration is typical of the upper Blue Nile tributary system of which this district is a part. The valleys and canyons are seasonally malarious, while the surrounding highlands are largely malaria free. It was reported by Sanford,[7] from the Mulu Farm Community which is situated in this territory, that the 1958 malaria outbreak was the worst ever experienced since the community was established 20 years before.

The experience at Mulu was but one of many hundreds of similar instances of fulminating outbreaks reported by anti-epidemic workers who distributed malaria drugs to villages and settlements throughout a vast region covering the upper Blue Nile River valley and its tributaries.

Malaria epidemics of varying intensity were also reported from the towns and villages located along the slopes of the eastern escarpment between elevations of 1,400 and 2,000 meters. The escarpment extends nearly 600 km north from Debra Sina in Shoa Province to Adigrat in Tigre Province. The main highway from Addis Ababa to Asmara, Eritrea, passes through much of the affected territory. Outbreaks of exceptional severity were observed at Lake Hiak (1,900 meters) near Dessie, Wollo Province, and in numerous other settlements, villages, and towns as far north as Makelle, Tigre Province.

Significantly, no malaria outbreaks were observed in the DDT residual sprayed villages of the ICA-assisted Kobo-Chercher Malaria Pilot Project at Alamata, but there were severe epidemics in adjoining unsprayed areas. Houses in the project area were sprayed in May and June of 1958 at the rate of 2 g of DDT per m^2 of wall space for the third annual cycle. The first was applied in 1956.

The occurrence of malaria epidemics in the upper Takazze River valley and its tributary valleys in Tigre and Beghemder Provinces was not properly evaluated because of the inaccessibility of the greater part of this large drainage area. However, investigations made near Makelle indicated that the incidence was unusually high.

On the basis of reports received from medical missionary stations and government hospitals in Sidamo, Kaffa, Arussi, Harar, and Gemu Goffa Provinces, malaria epidemics flared in the highlands of these provinces. The outbreaks were of sufficient intensity to attract the special attention of local officials and missionaries who requested

malaria drugs and the help of anti-epidemic workers.

Vector Prevalence

Although records of the prevalence of vector mosquitoes during the epidemic period are incomplete, there was enough entomological data accumulated to clearly show that *A. gambiae* was the only vector present in significant numbers. It was the only species seen in numerous adult collections taken from dwellings in the Lake Tana area, the Blue Nile valley and its tributaries, the upper Awash River valley and its tributaries, the malarious areas of the eastern slope of the plateau along the main highway from Addis Ababa to Asmara, and the upper Takazze River watershed.

The available collection data indicated that *A. gambiae* was abundant from June through September, 1958, with the numbers falling off rapidly thereafter. In November and early December, approximately 2 months following the end of the rainy season, it was a common experience to find from 50% to 75% of a village population sick with malaria, with an average vector density index of only two *A. gambiae* or less per dwelling compared to densities varying from 100 to 150 seen earlier in the season. Most of the mosquito breeding places had dried up with the passing of the rains in late September, and only remnants of the once large vector population remained, leading some inexperienced investigators to comment on what appeared to them to be a paradox of widespread malaria in the comparative absence of vectors.

Climatological Factors

In 1958, the rainfall, temperature, and relative humidity were significantly higher than in previous years, according to the meteorological reports and records compiled by Lissicine.[8] The span of years of the records varied greatly. A few station records, such as at Addis Ababa, extended over 35 years, while others in the provinces varied from 10 to 25 years, depending on the locations.

Reports showed that precipitation in 1958 was generally higher than that of any previous year on record, particularly in the highlands and on the western slopes. The high total, however, was not only the result of an abnormally high rainfall during the wet season, but was also due to rain falling in appreciable amounts during normally dry months before and after the regular wet season.

In Shoa Province rainfall exceeded that of all previous years on record. This was also the case in the provinces of Beghemder and Gojjam, where the epidemic was especially severe. In the highlands of Harar, Sidamo, Wollega, Illubabor, and Arussi Provinces, rainfall also exceeded all previous years on record.

In Wollo Province rainfall showed wide variations at different stations, but the average was greater than that of previous years.

In Tigre Province, which borders Eritrea on the south, the annual rainfall was generally less than average; however, the records at some stations exceeded previous years.

The average daytime temperature in 1958 was higher than in any previous year on record at most stations. Maximum daytime temperatures were also higher than usual, and nights were warmer. This was reflected in the limited and slight frost observed in 1958 as compared to previous years in which widespread heavy frost occurs regularly in most mountainous places and in many high valleys.

The yearly average relative humidity was higher in 1958 than in previous years on record. This is attributed to the fact that rainfall was distributed in the normally dry months, resulting in a generally higher relative humidity for all the months.

During the dry season, the average relative humidity at weather stations in the highlands is usually less than 50%. In 1958, it approached 60% and very often exceeded this figure. During the wet season relative humidity varied between 80 and 90%, which is normal for that period.

Plasmodia

P. falciparum predominated in all the epidemic areas investigated during the early stages and on through the time of peak prevalence in October. For example, out of 1,600 blood examinations of fever cases and cases with fever history made during the period September 1 through December 12, 1958, from representative epidemic areas, 40% were positive. *P. falciparum* accounted for 71% of the infections, *P. vivax* 22%, *P. malariae* 3%, and multiple infections 4%. There was no significant difference noted in the parasite incidence in the different age groups, thus indicating a lack of

communal immunity among the population examined.

It is interesting to note that Raffaelle et al.[9] reported the presence of two strains of *P. falciparum* from Ethiopia, the dominant classical strain and the less frequently observed Ethiopian or Abyssinian strain. The morphology of the latter is similar to that of the Malayan strain, which is characterized by a large chromatin dot and an intensely stained, above average size cytoplasm, according to Maegraith.[10] During the epidemic period, the Ethiopian strain was frequently noted in blood films examined by the ICA malaria survey team.

Morbidity and Mortality

Only estimates of mortality and morbidity are available for the epidemic, as there is no reliable system of disease reporting or registrations of death in Ethiopia. The available information is based largely on estimates made by Ministry of Public Health personnel and malaria advisors of the ICA and WHO who participated in an anti-epidemic campaign. In the campaign anti-malaria drugs were distributed to the epidemic areas by an organization of over 500 people who were drawn from the staffs of public health agencies, government hospitals, the ICA, WHO, missionary stations, the police, and the Ethiopian Red Cross. About four million chloroquine tablets were distributed in a period of 8 weeks.

The anti-epidemic workers were organized into teams of six to seven individuals. Each team was furnished with transportation to their assigned area, where they walked or rode (depending on the availability of local transportation) distributing drugs to the people in the villages. In some remote inaccessible areas, drugs were delivered by helicopter. As the peak of the epidemic had passed by the time anti-epidemic measures were undertaken, the main concern was to get the sick back on their feet so they could work in the harvest which had been neglected because of the epidemic. After some experience with different dosages, it was decided to administer a single immediate dose of 600 mg to all adults in villages where follow-up treatment was not possible. In places where health personnel were available locally, an additional 300 mg was administered daily for two days. Dosages for children and infants were appropriately reduced. It was observed that most individuals receiving the single 600 mg dose recovered sufficiently to work in the harvest within 3 to 4 days after treatment. This response was expected, as most of the sick were semi-immunes at the time they were treated.

Other sources of information on the epidemic were the unprecedented number of requests for medical assistance received by the Ministry of Public Health from provincial officials. The origin of the requests provided a basis for roughly determining the geographical distribution of the epidemic and, when pieced together, made possible a crude assessment of the relative severity of epidemics in different localities.

Of all the numerous estimates of morbidity and mortality received, those from Beghemder Province are the most complete and reliable because the provincial capital at Gondar is the site of the Gondar Public Health College, whose director and professional staff also provide public health services for the province as an additional duty. Some of the college staff, including senior students and interns, participated in the anti-epidemic campaign and contributed to the documentation of the epidemic, particularly around Lake Tana and adjoining districts. However, in the absence of a reliable population census of the various settlements and villages investigated, the vital statistics given here must be regarded as rough estimates, including those reported by qualified medical investigators.

Makonnen,[11] reporting on his investigations of nine villages near Lake Tana, indicated that out of an aggregate population of nearly 4,000, malaria was contracted by 3,000 people and 496 died during the first four months of an estimated 6-month epidemic period. Wasti,[12] reporting on nine districts near Lake Tana, with an estimated aggregate population of 170,000, gave the number of malaria cases as 83,000 and the deaths in excess of 5,000. Ryder[13], reporting on investigations made in 326 villages, with an estimated aggregate population of 131,000, gave the number of malaria cases as 75,100 in a 3-month period and the number of deaths attributed to malaria as 4,736. Schaller[14] reported 203 deaths and 959 clinically diagnosed malaria cases from seven villages (population unreported) in the vicinity of Lake Hiak, Wollo Province. The lake lies at 1,900 meters elevation. Kassaye,[15] reporting on investigations made in the Ganje district with an estimated population of 10,000, gave the number of malaria

cases as 5,287 from October 13 through December 17, 1958. The number of deaths attributed to malaria in this district was 1,064 from May through September, 1958.

The admission records for malaria compiled from two hospitals are given in Tables 1 and 2. Since diagnoses are made on the basis of clinical symptoms and since the hospitals serve only a small selective segment of the populations, the information shown must be regarded as no more than a crude assessment of the actual prevalence of malaria.

The Seventh Day Adventist Hospital at Debra Tabor, Beghemder Province, indicated a most unusual year for malaria. This small hospital, staffed by one European physician and nurse, is situated on the highlands west of Lake Tana at 2,600 meters elevation, which is well above the highest altitudinal limits of malaria transmission. In 1958 there were 2,780 malaria cases clinically diagnosed and treated. Of this total number, 2,513 were treated in October, November, and December, 1958. In the preceding 10-year period, which is as far as the records go, the number of malaria patients only exceeded 100 cases in 1953 and 1954, when 271 and 159 cases, respectively, were recorded. The higher totals in both these years coincide with an epidemic of malaria in the area of Lake Tana in 1953. According to the hospital records, none of the patients were residents of Debra Tabor. All of them traveled or were carried by friends and relatives up to the hospital from settlements and villages lying at elevations between 1,800 and 2,200 meters surrounding Debra Tabor.

At Debra Zeit, Shoa Province, 45 km southeast of Addis (elevation, 1,850 meters), 3,018 cases of malaria were treated by the European physician in charge at the government hospital during September, October, and November of 1958, out of a total of 4,094 malaria admissions for the year. This total compares with an average of 948 cases in 4 previous years on record.

The number of malaria cases and deaths attributed to the epidemic can only be estimated, as such records are not maintained except in the few hospitals and clinics which serve only a very small portion of the population. In the absence of reliable statistics, a rough estimate has been calculated as follows:

The population of Ethiopia is officially reported in excess of 18,000,000, of which an estimated eight to ten million live at risk to malaria. Of this number, it is estimated that at least three to three and a half million contracted malaria during the epidemic. The case fatality rate was known to have reached as high as 20% among the ill-nourished population of many villages where there were food shortages. In other districts, available information based on sample checks and inquiries indicated a case fatality of from 5 to 10%. Using a minimum of 5 deaths per 100 cases and three million cases as the total morbidity, the deaths due to malaria would amount to 150,000. Although the basis for determining morbidity and mortality is admittedly fragmentary from a statistical standpoint; nevertheless, when such information is weighed together with the subjective evidence, the estimated morbidity and mortality appear reasonably conservative.

The severity of the epidemic is most vivid to one reading the narrative descriptions contained

TABLE 1

Number of malaria cases diagnosed and treated in 1958 compared to the average of previous years on record as reported from two hospitals in Ethiopia

Name of hospital	City and province	No. malaria cases	Average no. malaria cases for prev. yr
Seventh Day Adventist	Debra Tabor, Beghemder	2780	126 (10 years)
Government	Debra Zeit, Shoa	4094	948 (4 years)

TABLE 2

Number of malaria cases diagnosed and treated by month of admission for 1958

Name of Hospital	J	F	M	A	M	J	J	A	S	O	N	D	Total
Seventh Day Adventist, Debra Tabor, Beghemder	20	6	20	19	30	51	58	22	41	549	1545	419	2780
Government Hospital, Debra Zeit, Shoa	42	51	133	33	90	130	155	98	465	1413	1140	344	4094

in the reports of anti-malaria epidemic personnel who worked in the rural areas conducting investigations and administering anti-malaria drugs to the people.

Taddesse,[16] in reporting on investigations made in late October in areas of the Blue Nile valley, wrote:

"It is hard to find a healthy person in a family. There are not enough healthy individuals remaining to fetch water for the sick. Many people are dying—as many as twenty persons out of a hundred sick. The crops which are unattended for lack of guards are being destroyed by baboons, wild pigs, birds, and other wild life."

Mamo,[17] in reporting on investigations made south of Lake Tana (Bahar Dar to Ganje), commented as follows:

"On October 13, we treated many malaria patients at Bahar Dar—everywhere we went we met cases of the same complaint. On the 14th, we went to Ganje, treating many patients on the way. As we approached the interior of Ganje, we came close to a big and deep river which we could not cross. We remained on this side of it and visited villages called Dishet, Wandata, and Sarualka. We saw many patients in their beds; practically everybody is sick. Over 500 died since the epidemic outbreak three months ago. The people said there is a slight epidemic every year, but such a serious outbreak was only known about thirty years ago."

Mogues,[18] conducting investigations in the north of Gojjam Province in late October, wrote:

"In each house we were able to find three or four patients who complained of subjective symptoms, such as chilling, severe headaches, sweating, pain in the back and extremities. The objective symptoms were jaundice, anemia, emaciation, high fever. After four or five relapses, the headaches and pain became unbearable for many patients, who then exhibited a muddling delirium with coma, ending in death. Most of the patients were between the ages of 5 and 20 years; second in frequency were infants and preschool children. Since they are far away from even the simplest clinic, which means no possibility of saving their lives, they are dying like bees in a smoked hive."

Many other observations along the same line were made by anti-epidemic personnel working in widely scattered areas of the country.

DISCUSSION

The extensive regional nature of the 1958 malaria epidemic is well established. It covered a region of not less than 100,000 square miles of the most heavily populated rural districts of Ethiopia and lasted from the end of June until the middle of December. It reached into high altitude zones rarely affected by the disease and there overwhelmed a large susceptible population whose last previous major contact with malaria probably goes back at least a decade, and probably longer. This is indicated by both the acute, widespread illness and high mortality observed and the information regarding the past history of epidemics related by the local people and Europeans of long residence in Ethiopia.

In some districts of Shoa and Gojjam Provinces the high mortality was undoubtedly aggravated by famine resulting from a depleted harvest in 1957. The resistance of a large population was thus ill-prepared to resist the onslaught of a virulent *falciparum* parasite. Although it is felt that such factors as undernourishment and parasite virulence may have exerted considerable influence on the mortality rate seen in some districts, the cause of the epidemic is attributed to unusual weather conditions in 1958, which favored propagation of the main vector and the parasite.

As mentioned previously, the weather of 1958 was unusual in that the recorded precipitation, temperature, and relative humidity were higher than past years on record in the highlands. Another significant difference recorded was in the distribution of rain, which in 1958 fell during normally dry months in appreciable quantities.

Considering these weather factors in the light of their effects on the propagation, longevity, and dispersal of the principal malaria vector mosquito, *A. gambiae*, it is evident that all three would be enhanced. Such factors would also favor development of the extrinsic cycle of the parasite *P. falciparum*. Taking this combination of vector and parasite operating under optimum conditions of development in a large, susceptible human population, we have then the essential ingredients for the generation and explosive extension of an epidemic such as was seen in 1958.

In explaining the cause of the regional epidemic in Northern Sind, India, in the autumn of 1929, Covell[19] stressed the importance of environmental factors favoring the propagation, longevity, and activity of the vector mosquito in an area inhabited by a population with a low-level communal immunity to malaria. Excessive rainfall which preceded the Northern Sind epidemic was attributed to be the precipitating

factor, since it produced favorable conditions for the development of the vector *Anopheles culifacies*.

We feel that these factors elucidated by Covell, favorable weather conditions for the vector, and lack of communal immunity among the population at risk, set the stage for the Ethiopian epidemic of 1958.

SUMMARY

An epidemic of malaria, covering about 100,000 square miles in four central provinces in the highlands of Ethiopia and portions of the highlands of five other provinces, was observed in Ethiopia between June and December of 1958. The altitudinal limits of the epidemic ranged approximately between 1,600 to 2,150 meters elevation, in which area the communal immunity of the habitants is low for the most part. The estimate of the number of malaria cases attributed to the epidemic is estimated to be not less than three million cases. Deaths from malaria may have exceeded 150,000.

Anopheles gambiae was the only vector involved. *Plasmodium falciparum* was the predominant malaria parasite. It accounted for the severe acute illness which characterized the clinical syndrome of most cases and the high mortality observed in many districts. Food shortages in 1958, due to 1957 crop failures in some areas, also indirectly contributed to high mortality.

The main precipitating cause of the 1958 epidemic appears to have been unusual weather conditions in the highland areas of Ethiopia. Rainfall exceeded all other previous years on record, and abnormally high atmospheric temperature and relative humidity prevailed.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. H. Larsen, Dr. F. B. Hylander, Dr. M. A. Zaphiropolous, Miss Abainesh Abebe, Ato N. G. Hanna, Ato G. Lemma, and members of the ICA-Ministry of Public Health malaria team who participated in the anti-epidemic investigations and control activities.

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EVALUATION OF TEST OF CURE PROCEDURES IN MICE TREATED FOR *PLASMODIUM BERGHEI* INFECTIONS

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Plasmodium berghei is invariably fatal to white mice once patency is established.[1, 2] Various antimalarials will, however, alter the course of the infection so that it becomes chronic or latent and the mice survive. What appears to be radical cure of the infection can be achieved by treatment with primaquine,[3] particularly if the treatment is timed to coincide with and reinforce the acquired immunity which is established by the 5th day after inoculation.[4] Such mice are relatively immune to subsequent challenge and this immunity has been the subject of several studies in our laboratory.[5, 6] For interpreting the basis of this immunity, it seemed important to ascertain whether primaquine completely eradicates the infection or whether the plasmodia are only reduced to submicroscopic levels and thus confer immunity as a result of premunition.

The present studies were undertaken in an effort to determine if, by using a more extensive test of cure procedures than had been utilized in our earlier studies, infections persisting after treatment would be detected which otherwise would have been overlooked. In earlier studies,[3] test of cure consisted of a series of thin blood smears every 5 days for approximately 1 month post-treatment, followed by splenectomy and additional blood smears. If all blood smears remained negative for plasmodia, the mice were considered to have been radically cured of their malaria infection. In the study reported here, the following procedures were employed: (a) a series of thin blood smears beginning on the day after cessation of treatment and continuing at intervals for 5 weeks; (b) subinoculation of blood into recipient mice; (c) splenectomy of donors with subinoculation of spleens into mice; and (d) sacrifice of donors with subinoculation of the total organ content into recipients. If no additional infections were encountered in primaquine-treated mice by these more extensive measures which encompass our former criterion of cure, it could be assumed that the animals have undergone radical cure and that the immunity acquired by mice following treatment with this drug is true residual immunity rather than premunition.

MATERIALS AND METHODS

The mice used in this experiment were young females of the CF_1 strain from Carworth Farms and weighed from 15 to 25 grams when inoculated. The KBG 173 strain of *P. berghei* obtained from the National Institutes of Health in 1958 was used. For infecting the 51 mice which were to undergo treatment, a standardized dose of 1 million parasites was inoculated intravenously. For determining parasitemias throughout the experiment, thin blood smears obtained from the tail vein were stained with Giemsa's stain. Each smear was examined with an oil immersion objective and a 10× ocular, traversing the smear once vertically. After determining that a parasitemia existed in each mouse on the day therapy was to begin, primaquine diphosphate was administered in the diet from days 6 to 11 inclusive at a level of 0.09% base. On the day after cessation of treatment, day 12, the blood of each mouse was examined for plasmodia and was examined thereafter every 5 days for 5 weeks, or 7 successive smears (8 in some instances). After examination of the series of post-treatment blood smears, it was found that approximately 40% of the mice still had a demonstrable parasitemia following treatment. Since animals with a parasitemia persisting after treatment had not been extensively followed in our laboratory before, it was decided that both groups of mice, the presumably "cured" and the known uncured, would be subjected to the same subinoculation procedures as outlined. In the former group, we hoped to ascertain whether the plasmodia had been completely eradicated in those mice with a complete series of negative smears following treatment. By subinoculating blood and tissues from chronically-infected mice into recipient mice, we hoped to see whether the strain of malaria had undergone any alteration in virulence due to chronicity, or whether it would still prove invariably fatal to

untreated mice. A further aim was to see whether splenectomy would produce relapses in "cured" and latent mice, and if so, of what magnitude.

After distinguishing, on the basis of the post-treatment blood smears, the presumably "cured" mice from the uncured mice, the following three subinoculation procedures were undertaken on both groups of animals:

1. *Blood subinoculation:* 0.25 ml of citrated tail blood from each treated mouse was inoculated intravenously into one recipient mouse.

2. *Splenectomy and subinoculation of spleens:* Donor mice were splenectomized 1 week after subinoculation of their blood into recipients. The total splenic tissue from each mouse was macerated in a tissue grinder in 0.5 ml of physiological saline. A volume of 0.5 ml of the splenic suspension was inoculated intraperitoneally into one mouse.

3. *Organ subinoculation:* Approximately 1 month after splenectomy, the donor mice were sacrificed. All tissues from each mouse, except the paws, skin, tail, digestive tract and bladder, were homogenized in 0.5 ml physiological saline in a Waring Blendor. The homogenate was strained through cheesecloth and 0.5 ml of the filtrate was inoculated intraperitoneally into one recipient mouse.

It was felt that one recipient mouse was sufficient for each subinoculation procedure since *P. berghei* is highly and reliably infectious. Blood smears were made every other day for 2 weeks on all recipient animals (with exception of certain of the organ recipients). Splenectomized donors were also examined parasitologically every other day for 2 weeks after splenectomy to detect relapse infections.

RESULTS

Treatment of a Primary Infection with Primaquine Diphosphate

In our earlier studies[3] primaquine diphosphate administered from days 6 to 11 after inoculation at a level equivalent to 0.09% base in the diet cured 5 of 5 mice. In later studies,[7] however, proportionately fewer mice were cured of a *P. berghei* infection by this treatment regime (7 of 41 uncured). In the present study, the series of post-treatment smears revealed that 40% of the 47 surviving mice still had a demonstrable parasitemia following treatment with 0.09% base primaquine (28 "cured," 19 uncured, 3 deaths presumably due to drug toxicity). An approximation of the amount of drug consumed by the individual groups of mice during the 5-day treatment period is shown in Table 1. There seemed to be little relation between the amount of drug consumed by the individual groups and the number of mice cured; *i.e.*, whereas 5 of 5 mice in cage 23b, no. 6–10, were cured by 72 mg/kg daily, only 1 of 5 mice was cured by the same amount of drug consumed by the group in cage 20b, no. 6–10. These two groups received more than the average daily drug consumption (66 mg/kg) which cured 5/5 mice in our earlier studies.[3] The deaths presumably resulting from drug toxicity occurred shortly after termination of treatment (on days 12, 13 and 17 after inoculation). All three of the mice had negative heart blood smears at autopsy.

Test of Cure Procedures on Presumably Cured Mice

The results of the test of cure procedures performed on the 28 presumably cured mice are shown in Table 2. Mice were arbitrarily placed in the "cured" group if the series of 7 smears taken at 5-day intervals after treatment remained negative for plasmodia. Each of the dash marks represents a negative blood smear obtained at the intervals shown in the table headings. As seen in Table 2, totally negative results followed subinoculation of blood, spleen, and organs from presumably cured mice into recipient animals. (Deaths occurring in recipients 1 to 2 days after organ subinoculation apparently were due to toxic effects of the inoculum.) Furthermore, splenectomy of the donor mice produced no parasitic recrudescences. Such findings indicate that these mice were cured of their malaria since no latent infections were uncovered by the additional infectivity procedures utilized subsequent to the series of post-treatment blood smears.

Test of Cure Procedures on Mice Uncured of a Primary Infection

By the series of blood smears taken after treatment, it was found that 19 mice were positive on one or more occasions following therapy. Of these 19, 4 died of the infection before the scheduled subinoculation of blood and organs into recipients. The remainder of the mice are represented in Table 3. It should be noted that four uncured mice had only a single positive blood

TABLE 1

Rates of cure relative to amount of primaquine consumed by mice

Cage* and group no.	Grams wt. of group		Grams food 5-day period	mg drug/kg daily	No. cured/no. treated
	Before treatment	After treatment			
Cg. 20a No. 1–5	102.5	92.0	31.3	57.9	3/5
Cg. 20b No. 6–10	110.0	90.0	40.0	72.0	1/5
Cg. 21a No. 1–5	113.0	94.5	30.6	53.1	3/5
Cg. 21b No. 6–10	106.0	90.0	31.5	57.8	2/4 + 1 drug death
Cg. 22a No. 1–5	109.5	91.5	34.4	61.5	4/5
Cg. 22b No. 6–10	103.0	92.0	32.0	59.0	2/5
Cg. 23a No. 1–5	102.5	92.5	30.8	56.8	3/4†
Cg. 23b No. 6–10	103.0	93.0	39.3	72.2	5/5
Cg. 24a No. 1–5	97.5	84.5	37.3	73.7	2/4 + 1 drug death
Cg. 24b No. 6–11	123.5	113.5	35.4	44.8	3/5 + 1 drug death

* A cage is comprised of two units, each containing 5 mice (except cage 24b), separated by a solid partition.

† One mouse escaped from this cage immediately after treatment so could not be evaluated for cure.

smear in the 8-smear series. These ranged from the 3rd to the 6th post-treatment smears (11 to 26 days after cessation of treatment). The 11 remaining mice were positive on a variable number of smears at various times post-treatment. Therefore, a series of smears appears to be necessary for ascertaining the presence or absence of parasitemia in mice so treated.

From the 15 infected mice mentioned above, 40 recipients were inoculated with either blood, spleen, or organs at the time intervals shown in Table 3. The signs in parentheses indicate the parasitemia state of the donor mice immediately before the test procedure. The results, as shown in this table, were as follows:

Blood recipients. Of 15 mice subinoculated with blood from uncured animals, 10 developed malaria. Six of the 10 died, 5 within 2 weeks and the 6th considerably later since it did not become positive for plasmodia until the 7th blood smear taken at 2-day intervals (no. 20-5). (As an additional check on possible undetected infections, heart blood smears were examined on all mice which died from miscellaneous causes within 1 year after termination of the experiment.)

Spleen recipients. Of the 15 mice which received spleens from uncured donors, 9 became positive for parasites. All of the 9 which developed malaria died from the infection, as indicated by the "D" letters in Table 3. The 10th recipient (no. 24-2) did not develop a demonstrable parasitemia. Spleen donors for the 5 other recipient mice which remained negative also failed to transmit the infection by other subinoculation methods and will be referred to later.

Organ recipients. Of the 10 recipients which received total organ subinoculations from uncured animals, 5 became positive. Four of the

TABLE 2

Response of mice inoculated with blood and tissues from donors assumed cured of Plasmodium berghei *malaria by primaquine therapy*

Mouse no.	Inoc. May 15 Sms. May 27–July 1 After treatment	Inoc. July 1 Sms. July 3–15 Blood recipients	Oper. July 8 Sms. July 10–22 After splenectomy	Inoc. July 8 Sms. July 10–22 Spleen recipients	Inoc. Aug. 12 Sms. Aug. 14–25 Organ recipients
20-2	7 neg. sms.	(−)*− − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
20-3	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
20-4	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
20-7	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
21-2	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
21-4	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
21-5	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
21-7	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead next morning
21-9	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-2	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-3	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-4	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-5	7 neg. sms.	(−) −Cannibalized	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-7	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
22-9	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-2	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-4	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-5	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-6	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-7	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-8	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-9	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
23-10	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
24-1	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead 2 days later†
24-5	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead 2 days later†
24-6	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) Dead 1 day later†
24-7	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −
24-9	7 neg. sms.	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − − − −

* Signs in parentheses indicate parasitemia state of donor mouse immediately before test of cure procedure.
† Smear of heart blood negative for plasmodia.

TABLE 3

Response of mice inoculated with blood and tissues from donors uncured of Plasmodium berghei *malaria by primaquine therapy*

Mouse no.	Donors inoc. May 15 Sms. May 27–July 1 After treatment	Blood recipients Inoc. July 3 Sms. July 5–17	Donors oper. July 10 Sms. July 12–24 After splenectomy	Spleen recipients Inoc. July 10 Sms. July 12–24	Organ recipients Inoc. Aug. 13 Sms. Aug. 12–24
Mice in category I					
20-8	− − + + + − − −	(−)*− − + + + + +	(−) − − + + + + +	− − + + + + + D†	(−) − − + + + D
21-3	− − − + − + − +	(+) − + + + D	(−) + + + + + − +	+ + + + + + D	(+) − − + + + D
22-10	− − − + + + − −	(−) − − + + + + + D	(−) + + + + + + +	− + + + + + + D	(+) − + + + + D
24-11	− − + + + − + +	(+) + + + + + + + + D	(−) + + + + + + +	+ + + + + + D	(+) − + + + + D
20-6	− − − + + + + +	(−) + + + + + + + +	(−) − + + + + + + + D	− − + + + + + D	Donor dead
20-10	− − − + − + − −	(−) − − − + + + +	(−) − + + + + + −	− + + + + + D	
22-8	− − + + + + + +	(+) + + + + + + + + D	D	+ + + + + D	Donor dead
24-10	− − + + + − − +	(−) − + + + + + D	(+) + + + + D	+ + D	Donor dead
Mice in category II					
20-5	− − + − − − − −	(−) − − − − − − + D	(−) − + + − − − +	− − + + D	(−) − − − − −
24-2	− − − + + − − +	(+) + + + + + + +	(−) − − − + − − −	− − − − − − − −	(−) − + − − −
Mice in category III					
20-9	− − − + − − − −	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − −
21-6	− + + + − − + −	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − −
22-1	− − − − − + − −	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − −
23-3	− − − + − − − −	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) D (bacteremia)
24-3	− − − + − − + −	(−) − − − − − − −	(−) − − − − − − −	− − − − − − −	(−) − − − − −

* Signs in parentheses indicate parasitemia state of donor mouse immediately before test of cure procedure.

† D = died from malaria infection.

5 died within 2 weeks; the 5th mouse (no. 24-2) developed a very transient parasitemia, demonstrable by only a single parasitemia reading of $+^{16}$ (16 parasites seen when traversing the slide once vertically). This was interesting, however, since the mouse which had received the spleen from this same donor 1 month earlier did not ever show parasites in its blood.

Of the 40 recipients inoculated with blood or organs from uncured mice, 24 developed malaria. Of these 24 which developed parasitemia, 5 mice survived—4 with pronounced parasitemias. Four of the survivors were blood recipients while the 5th survivor was an organ recipient inoculated from the same donor as one of the surviving blood recipients.

Splenectomy of uncured mice. As seen in Table 3, 14 mice known to be uncured by primaquine were splenectomized. The 15th mouse (no. 22-8) died immediately before splenectomy; its spleen was subinoculated into a recipient which succumbed to the resultant parasitemia. As indicated by the signs in parentheses in the column headed "After Splenectomy," mouse no. 24-10 was the only uncured mouse with a parasitemia at the time of operation; this animal developed a severe infection to which it succumbed in 8 days. Eight chronic mice with negative blood smears immediately before splenectomy whose blood recipients were positive, suffered relapses of varying intensity following operation. Of these, one died (no. 20-6), five developed pronounced but nonfatal parasitemias, one (no. 20-5) survived a less pronounced intermittent parasitemia and one (no. 24-2) had a scanty parasitemia detectable only on the 4th post-operative smear, 8 days after splenectomy.

Mice undergoing a spontaneous cure. As seen in category III of Table 3, there were 5 mice known to harbor malaria organisms after treatment with primaquine whose blood, spleen, or organs did not produce any infection in recipients. Furthermore, splenectomy did not evoke relapses in any of these 5 animals. These mice, although uncured by drug treatment, presumably underwent a spontaneous cure following the acquisition of immunity. Thus, the smears taken at 5-day intervals over a 5-week period immediately following treatment were the only means by which these mice were known to harbor latent infections, since none of the recipients inoculated with their blood or tissues developed patent infections.

As seen in Table 3, the infected mice fell into one of three categories: Included in category I are those mice from which subinoculation of blood, of spleen, and of organs all produced infections in recipients whenever employed. In category II, although subinoculation of blood transmitted the infection from both donor mice, in one instance the spleen recipient contracted malaria whereas the organ recipient did not. In the other instance, the converse was true with the organ recipient developing parasitemia and the spleen recipient, inoculated a month earlier, remaining uninfected. In category III are those mice in which no subinoculation procedure transmitted the infection; therefore, the donor animals apparently had undergone spontaneous cure.

DISCUSSION

The current study indicates that for detecting *P. berghei* infections persisting in mice following curative treatment a series of blood smears is adequate, provided such smears are taken at intervals sufficiently close and over a period sufficiently prolonged so as to detect fleeting parasitemias. Smears taken at 5-day intervals for 5 weeks proved satisfactory since no subsequent procedures uncovered infected mice in the group of 28 presumed cured on the basis of post-treatment smears. In addition, since all of the 15 infected mice were detected as uncured by means of serial blood smears, it appears that such test of cure procedures as blood subinoculation, splenectomy, etc. are excessive if the donors have been carefully followed by blood smears. The experiment obviously was not designed to compare the efficiency of the various infectivity procedures with each other, nor should it imply that thin blood smears are the only means whereby one is able to detect parasitemias persisting after treatment. Subinoculation procedures, if employed immediately following cessation of treatment and in place of donor smears, might prove of equal or greater diagnostic benefit (i.e., subinoculation of blood from donors at such a time might prove more rapid and practical for detecting persistent infections due to the volume of blood available for transmission as opposed to that for microscopic examination. One would not wish to

employ splenectomy routinely as a test of cure procedure on mice since removal of the spleen interferes with the host's immune response[5]).

An interesting feature of this experiment was the failure of five recipient mice to succumb to their malaria infections. This suggests that the plasmodia may have become altered in virulence during their residence in the donor animals. During several years of experimenting with the KBG 173 strain in our laboratory, untreated mice have always died once they developed a detectable parasitemia. The prepatent period could be lengthened by decreasing the inoculum but the parasitemia was ultimately fatal. However, the KBG 173 strain used in the present studies was reacquired from the National Institutes of Health in 1958. We have subsequently learned that the strain carried there had become less lethal to mice by 1959 and recovery of passage mice has occurred at another laboratory.[3] On the other hand, we had no survivals or noticeable prolongation of life in any of the animals used for routine passage of the organism obtained in 1958. In addition, control animals for the above experiments which were inoculated with one million organisms died in an average of 15 days, a figure comparable to that obtained in earlier studies. It seems probable, therefore, that the alteration in virulence noted in these experiments occurred in the chronically-infected mice in our laboratory rather than during the routine passage of the organism at the National Institutes of Health. Another possibility for survival of the five recipient mice in our experiments is passive transfer of immunity with the blood of the donors. It can be seen from inspection of Table 3 that whereas the blood recipients of mice 20-6, 20-8 and 20-10 all survived, the spleen recipients inoculated 1 week later all succumbed to the infection.

Another facet to this experiment which proved of interest was that of the apparent spontaneous cure in 5 donor animals. That mice with chronic infections may undergo spontaneous cure was suggested by Cox[9] who found that blood and minced spleen and liver from 4 mice, known to harbor infections after suppressive treatment, failed to transmit the infection to recipients inoculated 140 days later. The experiment reported herein, however, demonstrates that the time interval required for spontaneous cure may be much shorter than that suggested by Cox's experiment, since two of the spontaneously cured mice had positive smears only 1 week before their blood was subinoculated into recipients.

The two instances in which either spleen or organ subinoculation, but not both, transmitted the infection to recipients were interesting in view of the findings of Sergent and Ponce.[10] These investigators subjected rats, which had recovered from acute malaria infections, to various infectivity procedures for the purpose of detecting latent infections. They found that for detecting such infections by means of blood and organ subinoculation, results were dependent upon the amount and type of tissue used, since the parasites are unevenly distributed in the rat's organs. From the experiment reported herein, it would appear that cryptic infections persist in latent mice in much the same manner. Sergent and Poncet found that parasites were still detectable in the bone marrow and kidney of a rat 19 months after primary inoculation. Interestingly, these investigators remarked that, in spite of their scantiness, the parasites derived from latent infections exhibit a higher degree of virulence for recipient animals than do the large numbers inoculated from donors during acute phases of the infection. Such did not appear to be the case in the mouse experiment reported herein.

Another criterion of cure frequently employed in avian and simian malaria is that of evaluating the response of recovered animals to challenge inoculation. For example, Coggeshall and Kumm[11] found that, whereas one parasite is the minimal infective dose of *Plasmodium knowlesi* for monkeys, a monkey with a known chronic *knowlesi* infection was able to reduce a challenge inoculation of 17 billion parasites to submicroscopic levels within 24 hours. On the other hand, monkeys cured of a *P. knowlesi* infection by sulfanilamide were all susceptible to reinoculation. Somewhat inconsistent results have been obtained with this challenge technique using rodent malaria, however. Lapierre[12] found that when mice given suppressive treatment were challenged following recovery, one was completely refractory, two were reinfected with difficulty and one had a usual infection. In earlier experiments in our laboratory,[5, 6] cured mice, during the first week following challenge, had a parasitemia similar to that of control mice with no history of pre-

vious contact with the parasite. Following this initial period of infection, however, cured mice were able to bring the parasitemia under control whereas non-immune mice succumbed to the infection. Mice uncured of a primary infection by inadequate treatment with primaquine, however, did not behave uniformly following challenge, but rather behaved similarly to Lapierre's challenged mice.[12] When such animals were challenged, the response ranged from complete refractoriness in some to erratic, protracted parasitemias, and even death, in others.[7]

SUMMARY

In an effort to elucidate the state of immunity acquired by mice given curative treatment for a malaria infection, white mice infected with *Plasmodium berghei* were treated with primaquine diphosphate and subsequently exposed to various infectivity procedures for determining whether plasmodia had been completely eradicated or merely reduced to subpatent, chronic levels. Test of cure procedures included: (a) serial blood smears continuing at intervals for 5 weeks; (b) subinoculation of blood into recipients; (c) splenectomy of donors with subinoculation of spleens into recipient mice; and (d) sacrifice of donors with subinoculation of total organ content into recipients.

Blood examination was an adequate criterion for demonstrating radical cure in mice following primaquine therapy since all mice presumed cured on the basis of post-treatment blood smears failed to transmit the infection to recipients inoculated with their blood or organs. Splenectomy did not produce relapses in any of the cured mice.

An apparent attenuation of the infection was noted in recipients of blood and organs from treated animals.

One group of mice, with parasitemias on one or more occasions following therapy, apparently underwent spontaneous cure since they failed to transmit the infection to recipients by any infectivity procedure employed. Furthermore, splenectomy did not evoke relapses in these animals. Therefore, although serial blood smears taken after treatment are adequate for selection of mice uncured by drug therapy, the parasitemia state of such mice cannot be relied on for prognosticating the ultimate infection status of the animal and its consequent immune response to challenge infections.

ACKNOWLEDGMENTS

The writers wish to express their appreciation to Dr. Willard F. Verwey for his helpful suggestions and criticisms of this manuscript. The authors are also indebted to the Sterling-Winthrop Research Institute for the primaquine diphosphate used in these studies.

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SALMON DIET IN RELATION TO AMEBIC COLITIS IN THE DOG*

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Entamoeba histolytica in the human colon may be either a commensal or a pathogen.[1] Although its pathogenicity has not been fully demonstrated in experimentally infected human volunteers,[2] tissue invasion by *E. histolytica* has been repeatedly observed in a wide variety of experimental animals.[3]

In the dog, experimental infection can be established either by oral administration of cysts or by intracecal inoculation of trophozoites by canula through the anus. Infections thus established experimentally, and those which occasionally are acquired in nature, may be associated with ulceration of the cecum and colon. Soon after susceptibility to experimental *E. histolytica* infection had been demonstrated,[4, 5] it was observed that some dogs are refractory, and that a high percentage of the susceptible ones lose the infection spontaneously.[6] Later, however, it was discovered that dogs maintained on a diet of canned salmon were more easily infected, and the dysentery which almost invariably followed the inoculations developed more promptly and was more enduring and severe than that seen in dogs on normal diets.[7-9] Following this observation, the use of salmon diet to establish and maintain amebic infection in the dog became routine.[2, 10, 11]

Thompson[3] observed that the uninfected as well as the infected salmon-fed dogs used in trials of anti-amebic drugs consistently developed diarrhea, and that after 3 to 4 months some showed evidences of vitamin B-complex deficiency.[12-14] However, as the diarrhea and enhanced susceptibility to infection with *E. histolytica* occurred much earlier, the favorable effects of salmon diet on experimental amebiasis apparently were not related to the vitamin deficiency. Beaver *et al.*[2] observed diarrhea and dysentery in salmon-fed dogs in which *E. histolytica* infection could not be demonstrated. They noted in some cases that the bloody, mucoid stools possibly resulted from hookworm infection, or were related to an undiagnosed ulcerative condition of the mouth; in others, no cause for the dysentery was evident.

In the present study the results obtained in three experiments dealing with this problem are reported.

MATERIALS AND METHODS

Dogs to be used in the experiments were immunized against rabies and distemper, given standard anthelmintics, and observed for 2 to 4 weeks while maintained on a balanced diet of commercial dog food. All were apparently healthy, young adults weighing between 5.8 and 9.7 kg; both sexes were represented with a predominance of males. All were confined to cages of uniform size, 65 cm square and 70 cm high with coarse-mesh wire floors.

Repeated fecal examinations failed to reveal natural amebic infections. As only a few hookworm eggs were seen in the feces of dogs used in the first two experiments, no further anthelmintic treatments were given. However, numerous worms were found in some of these dogs at autopsy. All animals in the last experiment, therefore, were treated repeatedly until fecal examination by brine flotation failed to reveal hookworm eggs.

In a preliminary experiment, without controls, dogs were offered daily 1 pound of canned pink-salmon and water, but no other food. In the second experiment one group of dogs was fed exclusively on salmon and half of them were fed heavy inocula of *E. histolytica* cysts suspended in about 50 ml of milk. Salmon was given in the amount of 1 pound daily for 11 to 13 days before inoculations and continued until the dogs were sacrificed. Others were fed the normal diet, and some of this group were in similar manner given *E. histolytica* inoculations. The inoculations consisted of approximately 1 million washed

* Supported by research grants E-3032 and E-2550 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service.

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cysts obtained from four sources, all given together in the same suspension. About 90% of the inoculum consisted of equal numbers of cysts washed from fresh stools of three human carriers, the remainder were from cultures of one strain (C2). (The carrier of strain C2 had been under observation for about 3 years and had had no complaints which definitely could be attributed to the ameba. The other two carriers had been observed for less than 1 month; both had doubtful histories of mild symptoms of amebiasis.) In the third experiment salmon-fed dogs were given daily as much of the ration as they would eat during a 3-hour period. After about 2 weeks, half of them and an equal number on the normal diet were each fed 160,000 cysts of strain C2 obtained from cultures in Balamuth's encystment medium.[15]

Stools from inoculated animals were examined daily and the character of the feces as well as the microscopical findings were recorded. After being observed for 5 to 8 weeks, the animals were given lethal doses of Nembutal® either by intracardiac or intraperitoneal injection, and immediately after death the entire intestine was opened and tissue samples from different levels were fixed in 10% formalin. Samples of material from lesions, or suspected lesions, of the cecum and colon were examined for amebae microscopically and by culture in Balamuth's medium.[16]

RESULTS

Observations without Controls

Two of four dogs in the first experiment regularly ate the entire daily ration of salmon. The other two ate only sparingly throughout, and during the last 3 weeks progressively less. All dogs lost weight. After the first 2 or 3 days stools were predominantly soft, semifluid or fluid. Blood was seen in the stools of all dogs; amebae were not (Table 1).

In one of the dogs (P1) blood streaked mucus in soft stools appeared first on the 42nd day and on 5 occasions thereafter. This animal lost weight gradually; during the 9th week it also lost appetite and finally refused food and died on the 75th day, apparently of pneumonia. The intestine from duodenum to rectum contained abundant bloody mucus. The mucosa was congested, edematous, and friable, with numerous small hemorrhages that were confluent in several areas of the small intestine, cecum, and rectum. Seven hookworms were found in the small intestine, each with an area of bleeding at its site of attachment.

In two of the dogs (P2, P3) bloody stools first appeared at the end of the 4th week and continued almost daily until the 80th day when the normal diet was resumed. One of them (P3) lost appetite towards the end of the observation period and became so cachectic that death appeared imminent. When restored to normal diet it improved markedly within 1 week and was normal in appearance and behavior at the end of the 2nd week. In both animals the stools were normal after 48 hours.

The 4th dog began passing bloody stools on the 8th day and continued to pass small to large amounts of blood and mucus in mushy, semifluid stools almost daily. At times, it passed several stools per day which consisted of blood

TABLE 1

Summary of observations on four male dogs fed 1 pound of canned salmon daily

Conditions	Dog number			
	P1	P2	P3	P4
Days observed	75	80	80	96
Kg body weight at start/end	6.4/4.8	7.8/7.2	6.8/5.3	6.5/6.0
Day blood first seen in feces	42nd	29th	28th	8th
Bloody stools	6 days	Daily*	Daily*	Daily
Hookworms	7	Few eggs in feces	Few eggs in feces	26†
Fate	Died	Normal diet resumed	Normal diet resumed	Sacrificed

* With short periods of remission.

† 7 of which were in the large intestine.

TABLE 2

Observations on 18 dogs, all of which were fed canned salmon exclusively and half of which were inoculated with cysts of Entamoeba histolytica *given in food approximately 2 weeks after the beginning of salmon diet*

Dog		Kg body wt.		No. hook-worms	No. whip-worms	Days on salmon diet
No.	Sex	Start	End			. 5 . 10 . 15 . 20 . 25 . 30 . 35 . 40 . 45 . 50 .
Inoculated						
A 1	M	8.1	7.7	23	0	. . . . . *. . . . . B . . . . . S^{+}
A 2	M	7.7	7.2	39	4	. . . . . *. . . . . B . . . . . S^{-}
A 3	F	8.0	7.2	18	0	. . . B . . B B . . . . *. . B . . . . . B B B B B B B A A A A A A A A B A B A S^{++}
A 4	M	7.9	7.2	63	0	. . . . B B B B B B B B B *B B B B B B B B A A A A A A A A A A A A A A A a . B B S^{+++}
A 5	M	6.9	6.1	2	8	. . . . . *. . . . . A B A A B A A B A B A B A a a . a . . . A . B a . S^{++}
B 1	M	6.9	6.3	0	0	. . . . . *. . . . . A . A . B . a . . . a . . A . . S^{++}
B 2	M	5.8	5.5	2	0	. . . . . B *. B . . . . . B a . B . a . . a . . A . . a . . a S^{+}
B 3	F	6.1	5.7	0	2	. . . . . *. . . . . S^{++}
B 4	F	6.3	6.1	0	0	. . . . . *B . . B . B . . . . S^{+}
Non-inoculated						
A 6	M	8.1	7.6	15	59	. . . . . B . S^{+}
A 7	M	7.0	6.2	43	20	. . . . . B B . . . B . B . . B . B B B . B . B . B . B . . . . . S^{++}
A 8	M	6.8	6.6	40	0	. . . . . B . . . . . $S^{\pm}$
A 9	M	6.5	6.1	9	0	. . . . . $S^{\pm}$
A10	M	9.2	9.1	8	0	. . . . . $S^{\pm}$
B 5	M	6.5	6.3	1	0	. . . . . B B B B B . . B B . B B . . B . B . B . . B B . . S^{++}
B 6	F	6.0	5.8	1	0	. . . . B B B B B . B B . B B B . . . . . B B . . . . . B . B . . B . B B S^{+}
B 7	M	6.2	6.9	0	6	. . . . . $S^{\pm}$
B 8	F	5.8	5.2	0	4	. . . . . B . . . B . . . . B . . . . . B . . . . B . . . . . B . . . . . $S^{\pm}$

. = days on which stools were normal.
B = gross blood in stool.
A = gross blood and amebae in stool.
a = amebae in mucus without gross blood.
S = sacrificed.
* = inoculated with *E. histolytica* cysts.
$^{-}$ = essentially normal mucosa.
$^{\pm}$ = congestion and edema; hemorrhage and ulceration not evident.
$^{+}$ = petechial hemorrhage mild.
$^{++}$ = hemorrhage and erosion, no discrete ulceration.
$^{+++}$ = hemorrhage extensive; erosion and macroscopic ulceration present and apparently not associated with hookworms.

and mucus only. However, its appetite remained good and there was no marked deterioration in its physical condition. At autopsy 26 hookworms were found, 7 of which were in the upper colon. There was moderate congestion of the mucosa and evidences of bleeding at the sites of hook worm attachment, particularly in the colon.

Observations with Controls

The second and third experiments differed only in that in the third special effort was made to expel all hookworms before the experiment was started, greater amounts of salmon were offered, and the cysts used for inoculation were obtained exclusively from cultures. These experiments therefore are described together, the dogs in the two series being distinguished respectively by the designations A and B.

Eighteen dogs were put on a salmon diet and twelve on a normal diet. Nine from each group were given cysts of *E. histolytica* on the 12th, 13th, or 14th day. Thus there were 9 salmon-fed inoculated, 9 salmon-fed non-inoculated, 9 normal-fed inoculated and 3 normal-fed non-inoculated animals (Table 2).

On normal diet, stools of all 12 dogs were formed and almost without exception, normal. To facilitate searching for amebae the dogs of this group were purged on the 22nd and 25th days after inoculation. In the purged stools of two of the inoculated animals scanty blood was seen twice in one and once in another. Amebae were not found in the feces of these or any of the others of this group. None of the 12 dogs gained weight; 10 of them lost 0.1 to 0.3 kg (average, 0.23 kg).

On salmon diet (Table 2), all 18 dogs ate well throughout the experiment, generally consuming 1 pound or more of the ration daily. One (B7) differed from the rest in that it consumed greater amounts of salmon (up to 3 pounds per day), remained active in the cage and gained weight steadily. In all others there was slight to moderate loss of weight (12% in A5).

Amebic infections were demonstrable by fecal examination in 5 of the 9 inoculated salmon-fed animals beginning 6, 8, 8, 9, and 18 days after inoculation. There was an apparent relationship between the dysenteric state of the stools and the ease with which amebae could be demonstrated. The animals in which amebae were repeatedly demonstrated in large numbers (5) passed blood and mucus more frequently and in greater amounts than did those in which blood was seen infrequently (3) or not at all (1). However, it is to be noted that in 3 of the 5 infected animals bloody stools were observed prior to inoculation (A3, A4 and B2). Amebae were only occasionally demonstrable in the mucus of stools lacking macroscopic blood. In no instance were cysts detected.

Among the nine non-inoculated, salmon-fed dogs, three passed no macroscopic blood and two passed only one bloody stool although the stools usually were soft and were often diarrheic. Three of the remaining four dogs in this group passed bloody stools more or less daily for periods of 3 to 5 weeks; the fourth (B8) passed stools containing gross blood on 6 days at intervals of approximately 1 week beginning with the 2nd week of observation. One dog (B6) started passing blood after 5 days on salmon diet and continued intermittently to pass large amounts of bloody mucus, sometimes two or three times daily.

Post-mortem Observations

Hookworm infections. All of the dogs on salmon diet in series A harbored hookworms (Table 2). The dog most heavily infected (A4, with 63 worms) passed bloody stools most frequently, but there was otherwise no correlation between worm burden and frequency of bloody stools. Among the eight salmon-fed dogs of series B, five harbored no hookworms and the others harbored only one or two. Among the twelve dogs on normal diet six harbored 1 or 2 hookworms, two had 9 or 10, and four had 30 or more. Two of the last (with 30 and 58 hookworms) had passed small amounts of gross blood in fluid stools when purged.

Amebic infections. Trophozoites of *E. histolytica* were demonstrated post mortem in only one dog in which infection had not previously been detected by fecal examination, this being one of the normal-diet B-series dogs in which amebae were found in an area of the cecum where whipworms were attached. As well as could be determined by the methods employed, amebic infections were established in five of nine dogs on salmon diet and in one of nine on normal diet.

Gross lesions. The only gross intestinal lesions observed in the dogs on normal diet were those

produced by worms. Bleeding at the sites of hookworm attachment was in these cases mild. In the salmon-fed dogs the intestinal mucosa ranged from essentially normal in one (A2) to severely hemorrhagic in another (A4). In general the conditions observed in the opened intestine corresponded with those indicated earlier by the appearance of the feces. Animals having passed abundant bloody mucus in the stools generally had areas of hemorrhage in the colon and cecum. Areas of bleeding at the sites of hookworm attachment, particularly in the colon, were distinctly more conspicuous in salmon-fed dogs than in those fed normal diet. While the mucosa of the cecum and colon was congested, and hemorrhage was conspicuous in about half of the salmon-fed dogs, macroscopic ulcers were evident with certainty in only those animals in which hookworms were, or possibly were, responsible for them. However, the abundant mucus and blood adherent to the mucosa in approximately half of both the inoculated and non-inoculated salmon-fed animals was such as to suggest discrete areas of ulceration. The most distinctive macroscopic feature of the colon and cecum of these animals was the edematous, friable mucosal lining which could readily be drawn away from the deeper tissues by lightly brushing away the covering mucus. In three dogs (P1 and B6, uninfected; and A4, infected) the mucosa of the small intestine was likewise hemorrhagic and friable. In general, the condition of the mucosa appeared to be much the same in the salmon-fed dogs whether amebae were present or not. Considering each as a group, there was a marked difference between the gross appearance of the opened colon of salmon fed dogs and that of the dogs on normal diet.

Microscopic lesions. Among the dogs fed normal diet the only lesions seen in microsections of the intestine were those associated with hookworms or whipworms. In one dog (previously referred to) a colony of amebae was observed in the cecum along with several whipworms, but the trophozoites were confined to the lumen (Figure 1).

Among the salmon-fed dogs there was one (A4) in which amebae had invaded the deeper portions of the mucosa but had not penetrated the muscularis mucosae (Figure 2). In this and others of the inoculated dogs there were numerous small colonies of amebae among sloughed epithelial cells at the surface of eroded areas.

In all of the salmon-fed dogs in which blood and copious mucus were seen in the open colon, the histopathological findings, irrespective of the presence of amebae, included moderate to severe congestion and edema, mild to moderate infiltration of lymphocytes in the submucosa, extreme development of goblet cells and moderate to severe erosion of the surface epithelum (Figures 3 and 4). Hemorrhage into the tissues was seen infrequently.

DISCUSSION

In general, all salmon-fed dogs lost weight during the 5 to 7 weeks of observation. The exception was a small (6.2 kg) dog which gained 700 grams in 45 days. This animal was exceptionally active and friendly and maintained a vigorous appetite. Loss of weight in these animals should be expected, as salmon is lacking or inadequate in some important vitamins and minerals. However, weight loss possibly was due more to lack of appetite than to quality of the diet, and this in turn probably was due largely to confinement. The importance of this factor has been noted in experiments with kittens.[17] None of the dogs on the normal diet gained weight but their average weight loss was less than that of the salmon-fed group. Although no evidence of a particular vitamin deficiency was observed, it is recognized that the pathological conditions seen in the salmon-fed dogs might have been due to dietary deficiencies, as well as to other factors such as toxic substances in the canned salmon or alteration of the intestinal flora, alone or in combination.

There was a striking and prompt change in the character of the feces of salmon-fed dogs. In all instances stools became soft or semifluid, and usually within 1 or 2 weeks mucus became a conspicuous component. In a high percentage of the mucoid stools blood was grossly visible, sometimes as early as the 5th day on salmon diet. The bloody mucus possibly can be attributed in some of the dogs to hookworm infection and in others to amebic infection. In a number of cases, however, blood and mucus were equally conspicuous when amebae were absent and hookworms were either absent or few in number. The dysentery-like character of

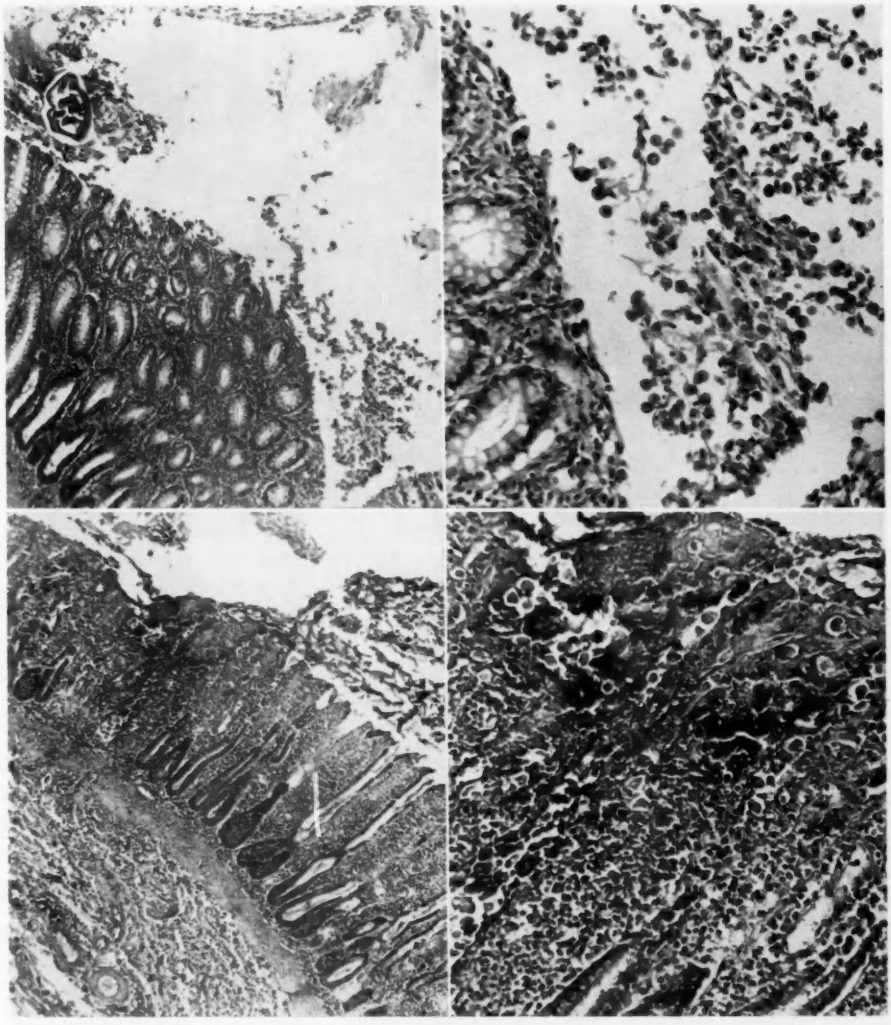

FIG. 1 A and B (upper). Cecum of dog on normal diet and infected with *Entamoeba histolytica*. The amebae have colonized an area inhabited by *Trichuris vulpis* where the worm, the amebae, or both appear to have produced shallow erosion of the mucosal epithelium. A, × 55; B, × 170.

FIG. 2 A and B. Colon of dog (A4) on salmon diet and infected with *Entamoeba histolytica*. The mucus contains sloughed mucosal cells; the eroded mucosa is infiltrated with lymphocytes and neutrophils; and the necrotic area is invaded by amebae. A, × 55; B, × 170.

the stools, therefore, must in some degree be attributed to the salmon.

As amebae were readily found in the stools of five of nine inoculated dogs on salmon diet and were not found in any of nine inoculated dogs on normal diet, despite the fact that infection was found at postmortem examination of one, it is not yet clear whether salmon diet rendered the dogs more susceptible to amebic infection or merely improved the conditions necessary for

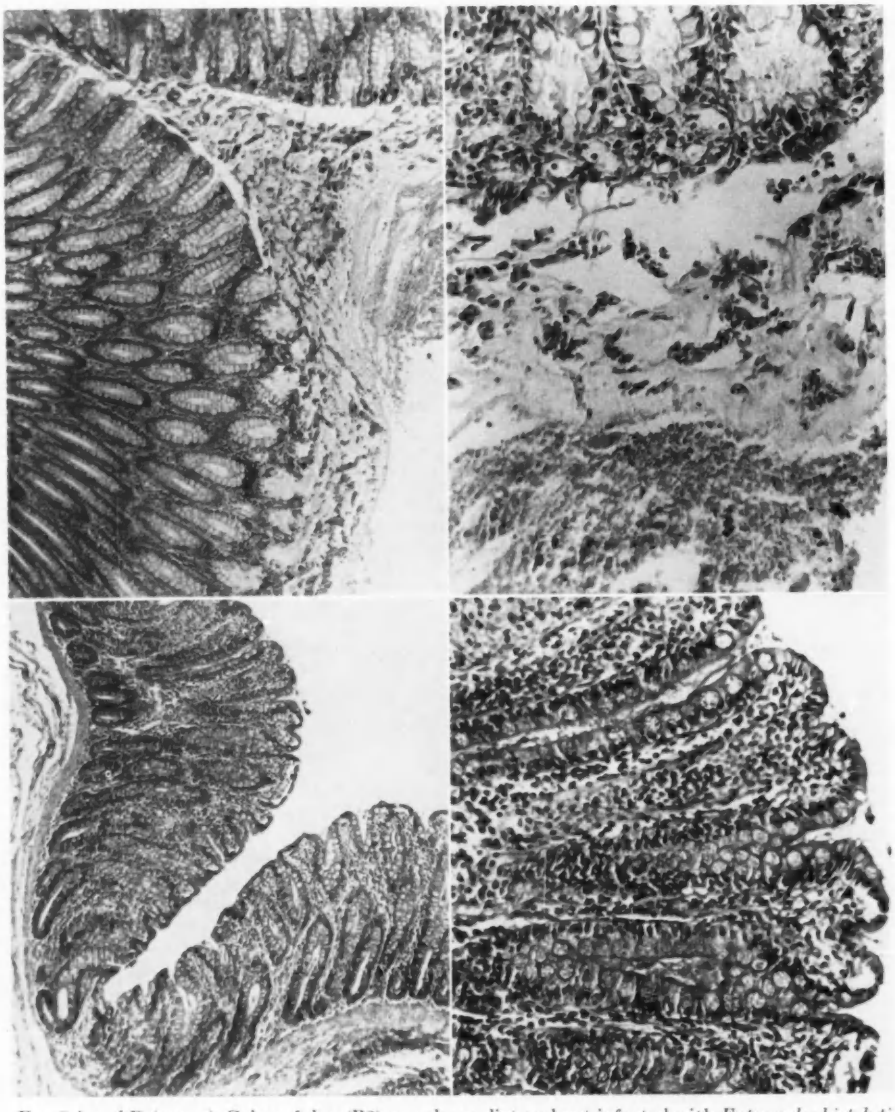

FIG. 3 A and B (upper). Colon of dog (B6) on salmon diet and not infected with *Entamoeba histolytica*. Goblet cells are numerous and active; the mucus covering of the mucosa contains masses of sloughed epithelial cells. A, × 55; B, × 170.

FIG. 4 A and B. Colon of dog on normal diet and not infected with *Entamoeba histolytica*. A, × 55; B, × 170.

detecting infections when present. As *E. histolytica* rarely encysts in the dog,[18] and trophozoites are not readily found in the dense fecal matter of the normal dog stool, or even in diarrheic (purged) stool if mostly fecal, the dysenteric quality of the stools of dogs on salmon diet undoubtedly serves to facilitate the detection of amebic infection by sweeping out and at the

same time preserving the motile amebae. Enormous numbers of active trophozoites containing red blood cells were often present in the bloody, mucoid stools. Occasionally they could be found in mucus when no gross blood was present.

Although several strains of amebae were used for infecting the dogs in the present study, pathogenicity was not clearly demonstrated. When amebae were present the appearance of the stools and of the opened colon and cecum would in most instances have been strongly suggestive of amebic dysentery. In one instance an open lesion of the mucosa contained amebae. In view of essentially identical observations in instances in which amebae could not have been involved, it might be concluded that the strains of amebae used in the present study contributed somewhat to the degree of pathological change, but were not pathogenic in the sense that they initiated a pathological process. The impression gained from the combined gross and microscopical observations is that in the salmon-fed dogs, in which there was erosion of the mucosa accompanied by flow of blood and tissue fluids into the lumen, the amebae colonized more readily in the intestine, and, once established, thrived on elements derived from the tissues.

It is to be noted that gross ulceration generally was not seen in dogs regularly passing dysenteric stools, the blood being in these cases from numerous small hemorrhages in the friable, eroded mucosa. However, the use of any firm instrument for irrigation or for aspirating material from the upper colon apparently would have been severely damaging and might have produced relatively large lesions.

In view of these findings, previously reported observations on amebiasis in salmon-fed dogs may require reinterpretation. In one report,[2] for example, it was stated that a strain of amebae apparently non-pathogenic in human volunteers "produced typical amebic lesions in dogs." It now would seem better to have stated in this instance that the amebae were present in lesions appearing to be typical of amebic colitis, not that the lesions necessarily were produced by amebae. In another study[10] the pathogenicity of 16 strains of *E. histolytica* was tested in 26 salmon-fed dogs that had been irrigated, inoculated and aspirated through the rectum. Because lesions were observed in the colon, all strains were considered to be pathogenic despite inability to demonstrate amebic infections in half of the inoculated animals. It is now evident that in these experiments and others[4, 8, 9, 11, 19, 20] in which dysentery was produced in salmon-fed dogs the amebae may have had little if any influence on the conditions observed.

SUMMARY

In three experiments the character of the feces and the condition of the colon and cecum were observed in 34 young adult dogs that had been placed on either a normal or salmon diet and, after 2 weeks, either inoculated orally with cysts of *Entamoeba histolytica* or not inoculated.

During the 34 to 96 days of observation, most of the dogs lost weight, those on the salmon diet to a greater extent than the others. No signs of vitamin deficiencies were observed. Twelve dogs on normal diet passed normal stools and displayed no intestinal lesions not attributable to worms; one of nine inoculated with *E. histolytica* acquired infection.

After 5 to 29 days, among 22 dogs on salmon diet, 12 regularly passed blood and mucus in fluid, often dysenteric, stools. Five of these dogs had been inoculated with *E. histolytica*, seven had not. In two of the salmon-fed dogs the stools were consistently normal and the intestinal mucosa also appeared to be normal; in the others, the mucosa was in some degree hemorrhagic and friable, with masses of sloughed epithelial cells in the covering mucus. *E. histolytica* infection was demonstrated in 5 of 9 inoculated dogs, but pathological changes seen in the colon and cecum were essentially the same irrespective of amebic infection. Bloody stools and bowel lesions in some cases apparently were in part due to hookworms, and amebae might have contributed to some of the changes seen; in most instances, however, the salmon diet appeared to have provided the chief causative factor. Thus, the results from earlier studies of amebic colitis in salmon-fed dogs may require reinterpretation.

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STUDIES ON COMPLEMENT FIXING ANTIGENS ISOLATED FROM *TRICHINELLA SPIRALIS*

I. Isolation, Purification and Evaluation as Diagnostic Agents

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Attempts to isolate complement fixing antigens specific for the diagnosis of trichinosis began in 1911. At that time, Strobel[1] demonstrated the presence of complement fixing antigens in a 0.1 N sodium hydroxide or antiformin extract of a pepsin digest of larvae infected rat muscle. Many subsequent studies have been reported and these have been excellently reviewed.[2, 3] Included in these studies were several attempts to improve the specificity and sensitivity of the isolated complement fixing antigens by subjecting them to physicochemical procedures.[4-6]

In general, the complement fixing antigens isolated by later investigators were usually sufficiently sensitive for the diagnosis of trichinosis. Complement fixing antibodies have been detected in sera of infected animals within one week after ingestion of the larvae.[6-8] The elapsed time between the ingestion of larvae and the appearance of antibody has been related to the severity of the infection.[9] In addition, antibodies have been detected several years after the initial infection.[10] Although adequately sensitive, these antigens were not specific for the diagnosis of trichinosis. Cross-reactions have been reported between trichinella antigens and sera from *Dipylidium canium*,[11] *Ascaris lumbricoides*,[12, 13] *Trichuris trichiura*,[14] *Necator sp.*,[13] and *Salmonella typhosa*[15-17] infections. Trichinella antisera also has been reported to react in agglutination tests with *Proteus X19* organisms.[15]

Extracts of *Trichinella spiralis* larvae were subjected to chemical fractionation, therefore, in an attempt to isolate a complement fixing antigen specific for the diagnosis of trichinosis. Such a purified antigen would also permit a study of the chemical structure of the antigen. The complement fixing system was chosen because of its sensitivity and also because the test could be standardized to give precisely reproducible results.

* This paper is taken in part from a dissertation submitted by the senior author to the Graduate School of Georgetown University in partial fulfillment of the requirements of the Ph.D. degree.

MATERIALS AND METHODS

Isolation and Purification of Antigens

Collection of larvae. T. spiralis larvae were obtained from the Laboratory of Immunology, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, and carried in rats in our laboratory. Trichinella larvae were isolated from the muscle of intragastrically infected rats by the method described by Bozicevich,[18] washed with saline until free of rat protein,[19] and then washed with distilled water, lyophilized, and stored at −40°C.

Isolation of antigens. The following extraction processes were performed with 100 mg of dried larvae. The procedure is outlined in Figure 1 and detailed below. All procedures were conducted in the cold with precooled reagents in order to minimize denaturation of proteins.

Weighed amounts (100 mg) of dried larvae were suspended in 10 ml of 0.15 M sodium chloride (pH 7.0) in a glass tissue grinder, and allowed to stand for several hours at 3 to 6°C during which time the larvae absorbed the solution and increased in volume. The swollen larvae then were ground for 5 minutes using a motor driven teflon pestle. The resultant suspension was allowed to extract overnight at 3 to 6°C, and then ground for another 5 minutes prior to centrifugation (1,000 G for 30 minutes). The supernatant solution, which contained the antigenic activity was decanted, and the residue washed with 2 ml of 0.15 M sodium chloride. The wash solution was combined with the original extract and the insoluble residue discarded. The combined extract and wash were centrifuged again at 2,000 G for 30 minutes to further clarify the solution before fractionation.

Fractionation of the antigens was accomplished

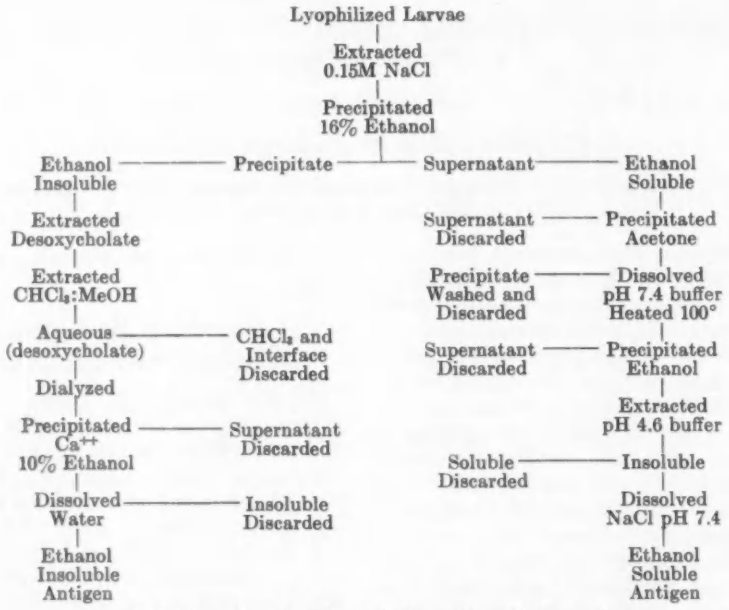

FIG. 1. Outline of method for isolating and purifying antigens

by adjusting the saline extract to pH 6.0 (Beckman Model G pH meter) with 0.1 N acetic acid and adding slowly with stirring 3 volumes of cold (−5°C) 24% ethanol. This gave a final ionic strength of about 0.04 with respect to sodium chloride, and an alcohol content of 16%.[20] The solution was permitted to stand overnight at −5°C to ensure complete precipitation of the insoluble antigenic material. The precipitate was recovered by centrifugation (1,000 G for 30 minutes) and washed twice with 2 ml quantities of 0.04 M sodium chloride containing 16% ethanol. The washings were combined with the ethanol soluble supernatant solution. The antigen soluble under these experimental conditions was designated as ethanol soluble (ES), and the precipitated material, the ethanol insoluble (EI) antigen. Each antigen was purified further by physicochemical methods utilizing processes which removed serologically inactive material.

Purification of the ES antigen. An equal volume of acetone was added to the supernatant solution containing the ES antigen. The solution was allowed to stand overnight at −5°C to completely precipitate the serologically active component. The precipitate was recovered by centrifugation (1,000 G for 30 minutes), and the supernatant solution, inactive in the complement fixation test, was discarded. Residual acetone was removed from the precipitate by a stream of air. The precipitate then was largely dissolved in a minimum volume (3 ml) of veronal buffer (VBS) pH 7.4*, and heated in a boiling water bath for 5 minutes. After cooling, heavy serologically inactive precipitate was removed by centrifugation (2,000 G for 30 minutes). The precipitate was washed twice with 1 ml portions of VBS, and the washings added to the decanted solution containing the ES antigen.

The antigen containing material was then concentrated by precipitation with an equal volume of 95% ethanol. The process was conducted overnight at −5°C. The precipitate was again recovered by centrifugation (1,500 G for 30 minutes), and the decanted supernatant was discarded. This precipitate was extracted three

* Prepared by dissolving 2.3 g 5,5′diethyl barbituric acid in 500 ml of hot distilled water, then adding 1.5 g sodium 5,5′diethyl barbiturate, 1.26 g sodium bicarbonate, and 41.90 g sodium chloride. Solution was cooled and diluted to 1 liter. This stock solution was diluted 1:5 with distilled water before using.

times, rapidly (2 to 3 minutes) and in the cold (0 to 3°C) with 2 ml of 0.1 N acetate buffer pH 4.6. The precipitate was recovered each time by centrifugation (1,500 G for 30 minutes) at 0 to 3°C. The acetate extract contained a precipitating antigen, as shown by gel diffusion,[23] but one that was inactive in complement fixation tests. The precipitate was dissolved finally in 10 ml of 0.15 M sodium chloride (pH 7.4). Aliquot portions were stored at −20°C without further treatment or after lyophilization. The antigenic activity of the material was not affected by freezing or lyophilization. After dialysis against distilled water, the yield of the lyophilized ES antigen was 2.6 to 3.2 mg.

Purification of the EI antigen. The ethanol insoluble precipitate was extracted with 10 ml of sodium desoxycholate solution (0.4% sodium desoxycholate, 0.1 M sodium chloride and 0.2 M sodium citrate) for 12 to 16 hours to solubilize the antigen. The resultant opalescent solution was treated by gently shaking for 10 minutes with 5 ml of chloroform-methanol (4:1). Centrifugation (1,000 G for 30 minutes) separated the emulsion into three distinct fractions: chloroform, a protein interface between the chloroform and aqueous phases, and the aqueous. The antigen containing aqueous phase (top) was removed by careful aspiration, and dialyzed for 24 hours against tap water to remove citrate. The solution was removed from the dialysis tubing, the volume measured, and sodium chloride added to make a 0.7% solution (W/V), which helped solubilize serologically inactive materials in the next step. The EI antigen was precipitated from solution by the addition of calcium ion (1.0 ml of 0.5 M calcium acetate) and ethanol (10% by volume). After standing overnight at 5°C, the precipitated antigen was sedimented by centrifugation (1,000 G for 30 minutes), dissolved in 4.0 ml of water, centrifuged again (1,000 G for 15 minutes) to remove insoluble substances, and decanted. The precipitate was washed once with 1.0 ml of cold distilled water and the wash added to the supernatant. To the decanted supernatant and wash solution 5 ml of 0.3 M sodium chloride was added to give a final salt concentration of 0.15 M. This antigen retained its activity in complement fixation tests for over 9 months when stored at 3 to 6°C. The serologic activity of this antigen in complement fixation tests was not affected by dialysis, but was decreased considerably by lyophilization or freezing. The yield of the lyophilized EI antigen was 0.5 to 0.8 mg following dialysis against distilled water.

Test Sera and Serologic Procedures

Human sera. Sera designated as healthy were obtained from candidates for appointment to the U. S. Military Academy, West Point, New York, who had passed a rigorous physical examination. Sera from leutic patients were all reactive in treponemal immobilization tests. The sera from patients with *Schistosoma mansoni* infections were obtained from native Puerto Ricans. Sera from persons with other parasitic diseases were obtained from the 406th Army General Medical Laboratory in Japan, and from the Communicable Disease Center, Atlanta, Georgia. The trichinella sera were from individuals whose diagnosis was documented by muscle biopsy. The diagnoses of other parasitic diseases were confirmed by the demonstration of eggs or organisms in excreta.

Infection of guinea pigs with T. spiralis *larvae.* Five thousand to six thousand gram guinea pigs were infected intragastrically with 3,000 to 5,000 motile larvae. Weekly bleedings were taken from the heart, and the sera stored at −20°C until tested for antibody content.

Inoculation of rabbits with bacteria. Rabbits, 7 to 9 lb, were inoculated intravenously 3 times at weekly intervals with approximately 1×10^8 formaldehyde killed *Salmonella typhosa* and other organisms possessing antigens common with *S. typhosa: Salmonella typhosa* strains O 901, H 901, and Ty 2, *Salmonella paratyphosa* A, *Salmonella paratyphosa* C, ETS 32 (Vi containing), *Paracolobactrum ballerup*, *Salmonella virginia*,[24] and also *Shigella dysenteriae*.[22] Two weeks after the last injection, the rabbits were exsanguinated from the heart and the antisera stored at −20°C. The agglutination titers of the antisera were all greater than 1:1024.

Serologic tests. Complement fixation tests were performed using a standardized method based on the quantitative procedure described by Kent *et al.*[23] The reagent diluent was triethanolamine buffered sodium chloride solution[24] containing Mg^{++} and Ca^{++} in concentrations optimal for immune hemolysis.[25] The standardization of reagents was performed as described by Chaffee *et al.*[26] Readings were made visually by comparison with prepared standards.

TABLE 1

Effect of progressive purification on serologic activity of ethanol soluble antigen in complement fixation tests with homologous human antiserum

Fractions	Wt. of fraction mg/100 mg larvae	Antigen diluted 1:					
		5	10	20	40	80	160
Saline extract	10.0–10.5	4*	4	4	4	3	1
16% ethanol	7.4– 8.1	4	4	4	4	3	1
Acetone ppt.	6.1– 6.5	4	4	4	4	3	–
Heated 100°C	4.6– 5.2	4	4	4	4	2	–
Insol. pH 4.6	2.6– 3.2	4	4	4	4	2	–

* Numerical value represents degree of complement fixation; 4, complete fixation; the negative sign (–), no fixation.

The macroscopic agglutination tests were performed as outlined in the Army Technical Manual 8-227.[17]

Absorption studies. Sera was absorbed with an equal volume of concentrated ES antigen (antigen extracted from 50 mg of organisms contained in 1 ml of solution) for 2 hours at 37°C and 16 hours at 3 to 6°C, and the antigen-antibody complex removed by centrifugation. Absorption with the *S. typhosa* and Proteus antigens were conducted for 4 hours at 52°C and 16 hours at 3 to 6°C prior to centrifugation. The bacterial antigens and antisera were obtained from the Biological Depot, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research.

RESULTS

Serological activity of various fractions. Purification of the ES and EI antigens was achieved with practically no loss of serologic activity. Each step in the procedure was chosen so as to retain maximum serologic activity during the removal of serologically inactive material. Table 1 shows the serologic activity of various fractions of the ES antigen against serum from a documented case of human trichinosis. The serum, 120957, was diluted 1:16. Undiluted antigen, in all cases, represented that amount of antigen derived from 10 mg of dried larvae and contained in 1 ml of solution. As can be seen, serologically inactive material was lost in each step, and the final fraction, which contained only 2.6 to 3.2% of the starting weight of dried organisms, retained essentially all the serologic activity of the original extract.

The EI antigen was not amenable to a similar stepwise study because of the presence of citrate which interfered in the testing procedure by chelation of cations necessary for the action of complement. The serologic activity, therefore, of only the initial saline extract and the final product was compared in complement fixation tests (Table 2). The serum, 50157, was from a patient with suspected trichinosis, and reacted with the EI antigen but not with the ES antigen. The serum was used at a dilution of 1:5. The purification resulted in a marked decrease in the amount of material with no change in serologic activity.

TABLE 2

Effect of purification on serologic activity of ethanol insoluble antigen in complement fixation tests with serum 50157

Fraction	Wt. of fraction mg/100 mg larvae	Antigen diluted 1:				
		2.5	5	10	20	40
Saline extract	10.0–10.5	4*	4	4	3	–
Purified antigen	0.5– 0.8	3	4	4	2	–

* Numerical value represents degree of complement fixation; 4, complete fixation; the negative sign (–), no fixation.

Ultra-violet absorption. In addition to their distinct physicochemical properties, which were utilized in separating and purifying them, the ES and EI antigens also exhibited marked differences in their absorption of ultra-violet light, serologic reactions, and absorption of antibodies. The ultra-violet absorption spectra of the antigens was examined between 200 and 320 mμ using a

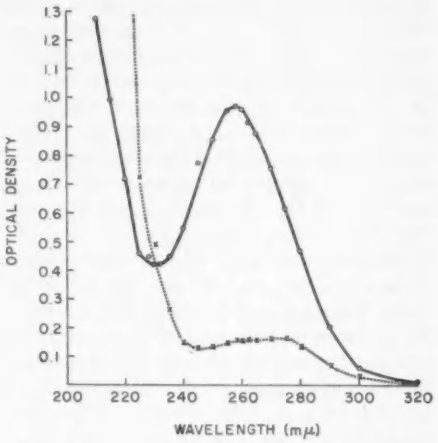

FIG. 2. Ultra-violet absorption spectra of the ethanol soluble (X-----X) and ethanol insoluble (O——O) antigens.

Beckman DU spectrophotometer. The optical densities obtained with 80 μg of the EI antigen and 160 μg of the ES antigen dissolved in water are shown in Figure 2. The absorption spectra for the antigens differed both qualitatively and quantitatively. The curve for the ES antigen revealed only broad, gradual changes in slope, similar to that exhibited by conjugated proteins other than nucleoproteins. The absorption curve for the EI antigen, conversely, exhibited a well defined minimum absorption at 230 mμ, and a sharp maximum at 258 mμ. It was very similar to that found with calf thymus deoxyribonucleic acid.[28]

Optimal dilution and reactivity. The antigens were also compared in complement fixation tests as to their optimal dilution and reactivity with selected sera. The optimal dilution of an antigen was defined as that amount of antigen which elicited a maximum reaction with a threshold concentration of antibody. The optimal dilution

TABLE 3

Reactivity of ethanol soluble (ES), ethanol insoluble (EI), and Witebsky (Wy) antigens with documented and suspected** trichinosis sera*

Serum	Antigen	Ant. dil'n	Results*** with serum diluted 1:						
			2	4	8	16	32	64	128
120957*	EI	1:5	4	4	4	4	4	3	2
	Wy	1:2	4	4	4	4	3	1	–
	ES	1:30	4	4	4	4	4	3	–
	Wy	1:20	4	4	4	4	4	2	–
50157**	EI	1:5	4	4	4	4	4	3	1
	Wy	1:2	4	4	4	4	4	3	1
	ES	1:30	–	–	–	–	–	–	–
	Wy	1:20	–	–	–	–	–	–	–
30460**	EI	1:5	3	4	4	4	4	2	1
	Wy	1:2	3	3	4	4	4	1	–
	ES	1:30	–	–	–	–	–	–	–
	Wy	1:20	–	–	–	–	–	–	–
20656**	EI	1:5	4	4	4	4	3	2	–
	Wy	1:2	4	4	4	4	3	2	–
	ES	1:30	–	–	–	–	–	–	–
	Wy	1:20	2	1	–	–	–	–	–
93057**	EI	1:5	3	4	4	4	4	2	–
	Wy	1:2	3	3	4	4	4	2	–
	ES	1:30	3	2	–	–	–	–	–
	Wy	1:20	2	1	–	–	–	–	–

*** Numerical value represents degree of complement fixation; 4, complete fixation: the negative sign (–), no fixation.

for the ES and EI antigens was determined by titrating twofold serial dilutions of antigen against twofold serial dilutions of trichinella antisera diluted in pooled normal sera.[29] With most sera, the optimal dilution for the ES antigen was 1:30, for the EI antigen 1:5. The EI antigen did not react at a dilution of 1:30.

The antigens also reacted differently with selected sera from documented and suspected cases of human trichinosis (Table 3). For comparison, parallel tests were run using antigen prepared by the method of Witebsky *et al.*[4] This antigen was used in routine testing for trichinosis at two dilutions, 1:2 and 1:20, to avoid possible loss of activity resulting from zoning.[10] It should be noted that the ES, EI and Witebsky *et al.* antigens reacted with sera from 15 documented cases of human trichinosis and all experimentally infected animals. Each antigen reacted strongly with serum 120957, obtained from a biopsy positive case of human trichinosis. Serum 50157, on the other hand, yielded a high serum titer with the EI antigen, but did not react with the ES antigen. Sera 30460 and 20656 gave similar results, while serum 93057 gave a strong reaction with the EI antigen but a comparatively weak reaction with the ES antigen. It was noteworthy that the antigen prepared by the method of Witebsky *et al.* at a 1:2 dilution reacted like the EI antigen, and at 1:20 like the ES antigen. From these results, it appeared that the ES and EI antigens reacted with different antibodies, and that the antigen prepared by the method of Witebsky *et al.* probably contained both the ES and EI antigens. The observed differences in reactivity of the different dilutions of the Witebsky *et al.* antigen can be explained by the

TABLE 4

Serologic activity of trichinella antisera after absorption with ethanol soluble (ES) and ethanol insoluble (EI) antigens

Antigen	Serum 90357														
	Unabsorbed					ES absorbed					EI absorbed				
	8	16	32	64	128	8	16	32	64	128	8	16	32	64	128
ES	4*	4	2	–	–	–	–	–	–	–	4	4	4	2	–
EI	4	4	4	4	4	4	4	4	4	2	4	4	4	4	3
Saline	–	–	–			–	–	–			4	4	4		
	Serum 100559														
	Unabsorbed					ES absorbed					EI absorbed				
	8	16	32	64	128	8	16	32	64	128	8	16	32	64	128
ES	4	4	4	2	–	–	–	–	–	–	4	4	3	–	–
EI	4	4	4	4	4	4	4	4	3	2	4	4	4	4	3
Saline	–	–	–			–	–	–			2	2	±		
	Serum 50157														
	Unabsorbed					ES absorbed					EI absorbed				
	4	8	16	32	64	4	8	16	32	64	4	8	16	32	64
ES	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–
EI	4	4	4	4	3	4	4	4	3	3	4	4	4	3	2
Saline	–	–	–			–	–	–			3	1	–		

* Numerical value represents the degree of complement fixation; 4, complete fixation; the negative sign (–), no fixation.

presence of two antigens each having a different optimum dilution.

Absorption studies. Absorption studies employing the ES and EI antigens also illustrated their dissimilarity. Human trichinella antisera were absorbed with each antigen for 1 hour at 37°C and overnight at 3 to 6°C. An unabsorbed serum specimen was included as a control in which antigen was replaced by saline. Table 4 summarizes the results. Absorption of trichinella antisera 90357 and 100559 with the ES antigen resulted in considerable reduction of the antibody titer to the ES antigen in complement fixation tests. In contrast, the titer to the EI antibody was not significantly reduced in these sera or with serum 50157, which did not react with the ES antigen in complement fixation tests. The EI antigen, on the other hand, did not form antigen-antibody complexes which could be sedimented by centrifugation. Soluble complexes were formed, however, as shown by the reactivity of the saline control. The results demonstrated that the ES and EI react with different antibodies, and also that they may form different types of antigen-antibody complexes.

Sensitivity, specificity and usefulness in diagnosis. Further differences between the ES and EI antigens were noted when the antigens were evaluated in serologic tests to determine their usefulness in the diagnosis of trichinosis. Both antigens reacted with all tested sera from documented cases of human trichinosis and laboratory animals infected with *T. spiralis* larvae. However, the sensitivity and specificity of the antigens were markedly different when compared in parallel tests with sera from both trichinella and non-trichinella infections.

The sensitivity of the ES and EI antigens were studied in tests with sera collected throughout the course of human and animal trichinella infections. The results found with sera from selected human cases of trichinosis are summarized in Table 5. Antigen dilutions found to be optimal were used: 1:30 for the ES antigen and 1:5 for the EI antigen. It was noted with patients 1 and 3 that antibodies to the EI antigen preexisted or appeared earlier in the infection. Sera from patients 1 and 2 showed that antibodies to the EI antigen persisted longer than those to the ES antigen. In the case of patient 1, antibodies to the EI antigen were still detectable after about 2 years while those to the ES antigen were no longer detectable. In all cases, the serum titers found with the EI antigen were higher.

TABLE 5

Reactivity of sera from human trichinosis in tests with ethanol soluble (ES) and ethanol insoluble (EI) antigens

Patient	Antigen	Date of specimen	Results* with sera dil'd 1:					
			2	4	8	16	32	64
1	ES	3/15/57	−	−	−	−	−	−
		4/12/57	4	4	4	4	2	−
		9/20/57	4	4	4	3	−	−
		1/13/59	±	−	−	−	−	−
	EI	3/15/57	4	4	4	4	2	−
		4/12/57	4	4	4	4	4	4
		9/20/57	4	4	4	4	4	4
		1/13/59	4	4	3	1	−	−
2	ES	7/17/58	4	4	1	−	−	−
		10/1/58	1	±	−	−	−	−
		11/12/58	−	−	−	−	−	−
	EI	7/17/58	4	4	4	4	4	3
		10/1/58	4	4	4	4	2	−
		11/12/58	4	3	2	−	−	−
3	ES	7/17/58	−	−	−	−	−	−
		8/18/58	4	4	4	4	2	−
		9/16/58	4	4	4	4	1	−
	EI	7/17/58	4	4	3	2	±	−
		8/18/58	4	4	4	4	3	2
		9/16/58	4	4	4	4	4	3

* Numerical values represent degree of complement fixation; 4, complete fixation; the negative sign (−) no fixation.

Comparable results were found with sera from 12 guinea pigs infected intragastrically with *T. spiralis* larvae. Only three of these animals survived 6 weeks or longer; the deaths resulted from either the trichinella infection or cardiac punctures. The results given in Table 6 are representative of these found in this study. As with human trichinosis, it was noted that antibodies to the EI antigen were detected earlier in the infection, at about 7 days. Antibody titers to the EI antigen also persisted at higher levels throughout the study. Antibodies to the ES antigen, on the other hand, were detected only later in the infection, and in the three animals that survived 6 weeks or longer, dropped below detectable levels during the experiment. The

TABLE 6

Reactivity of serum from Trichinella spiralis infected guinea pigs with ethanol soluble (ES) and ethanol insoluble (EI) antigens

Antigen	Days after infection	Results* with guinea pig serum dil'd 1:					
		2	4	8	16	32	64
ES	0	–	–	–			
	7	–	–	–			
	14	3	1	–	–	–	–
	21	4	3	2	–	–	–
	28	3	2	–	–	–	–
	42	–	–	–	–	–	–
	56	–	–	–	–	–	–
EI	0	–	–	–			
	7	2		–			
	14	4	4	4	3		–
	21	4	4	4	4	3	
	28	4	4	4	3	1	–
	42	4	4	4	2	1	–
	56	4	4	3	2	1	–

* Numerical value represents degree of complement fixation; 4 complete fixation; the negative sign (–), no fixation.

TABLE 7

Reactivity of ethanol soluble (ES) and ethanol insoluble (EI) antigens in tests with non-trichinella sera

Diagnosis	Number sera tested	Antigen					
		ES			EI		
		R*	wr	–	R	wr	–
Healthy	102			102		4	98
Syphilis	27		1	26	9	6	12
Schistosomiasis†	26		2	24	10	8	8
Echinococcosis‡	8			8			8
Trichuriasis	10			10			10
Leishmaniasis§	5		1	4		1	4
Filariasis‖	6			6	2		4
Hookworm	5			5	2		3
Fascioliasis	1			1			1
Ascariasis	4			4			4
Trichstrongyliasis¶	6			6			6

* Results classified as R (reaction 4+, 3+, 2+); wr (weak reaction 1+, ±); or – (no reaction) with undiluted sera.

† *Schistosoma mansoni.*

‡ *Echinococcus granulosus.*

§ *Leishmania tropica.*

‖ *Acanthocheilonema perstans.*

¶ *Trichstrongylus sp.*

EI antigen gave higher titers with all studied sera. Peak titers occurred with either antigen at about the 21st day after infection.

The specificity of the ES and EI antigens was studied by comparing their reactivity in diagnostic tests with non-trichinella sera. The results are summarized in Table 7. The ES antigen proved to be quite specific for trichinosis. It yielded only 4 week reactions with the 200 non-trichinella sera tested. The EI antigen, on the other hand, gave 23 reactions and 19 weak reactions with the tested sera. In comparative studies with an antigen prepared by the method of Witebsky *et al.*,[4] which appeared to contain both the ES and EI antigens (Table 3), the reactions obtained were similar but weaker than those from the EI antigen. The antigen of Witebsky *et al.*,[4] used at dilutions of 1:2 and 1:20, gave 10 reactions and 33 weak reactions with the non-trichinella sera tabulated in Table 7. In addition to the tabulated results, other serum specimens were also studied. These included 7 sera from humans with protozoan infections: 2 *Giardia lambia*, 1 *Endamoeba histolytica* and 4 *Endolimax nana* which did not react with either antigen. Five sera from humans with systemic lupus erythematosus did not react with the ES antigen, but 2 of them gave reactions and 2 others weak reactions with the EI antigen. The results of this study indicated, therefore, that only the ES antigen could be considered specific for the diagnosis of trichinosis.

Further investigations were conducted to study the specificity of the ES and EI antigens; in particular, the cross-reactivity between trichinella antigens and typhoid antisera, and conversely, between typhoid antigens and trichinella antisera reported by other workers.[15-17] To investigate the possibility of cross-reactivity between trichinella antigens and typhoid antisera, high titered sera were prepared by inoculating rabbits with formaldehyde-killed *S. typhosa* and other organisms containing O, H, and Vi antigens common with *S. typhosa*. These antisera were tested in complement fixation tests with the ES and EI antigens. All preinoculation control sera reacted with the EI antigen, but no rise in titer was observed as a consequence of immunization. The ES antigen gave no reactions with the controls or the antisera. In addition to the rabbit antisera,

single specimens of anti-typhoid human and mouse serum did not react with either the ES or EI antigens in complement fixation tests.

The possible cross-reactivity between *S. typhosa* antigens and trichinella antisera was also investigated. Sera from 5 documented cases of human trichinosis and 8 trichinella infected guinea pigs were titrated in agglutination tests against *S. typhosa* antigens, O, H, and Vi. The results indicated that there are antibodies in sera from human trichinosis that cross-react with one or more of the *S. typhosa* antigens, but they are not present, at least in detectable amounts, in all trichinella sera. The typhoid titers, in the 3 sera that reacted with the *S. typhosa* antigens, rose simultaneously with the trichinella titer, remained elevated for about a month, then returned rapidly to a base line level. It should be noted, however, that sera from guinea pigs infected with *T. spiralis* larvae did not react with the *S. typhosa* antigens.

Absorption procedures were then performed to study further the cross-reactivity existing between the etiologic agents of typhoid fever and trichinosis. Two sera from trichinella patient 4 were absorbed with the ES antigen and the *S. typhosa* O antigen. The EI antigen was not included because it did not form precipitable complexes. The typhoid O antigen did not absorb antibodies that reacted in complement fixation tests with the ES antigen. The ES antigen, on the other hand, absorbed antibodies that reacted in agglutination tests with the typhoid O antigen. The absorption studies indicated that reactivity between typhoid antigens and trichinella antisera was non-reciprocal, or demonstrable only in agglutinating systems.

Cross-reactions, which have been reported, between *Proteus* OX19 antigens and some sera from trichinella infected persons[15] were not found with the above studied human sera. In addition, the ES antigen did not absorb antibodies from *Proteus* X19 antisera when studied in agglutination tests.

DISCUSSION

The complement fixing antigens obtained from trichinella larvae were solubilized readily by a single extraction with 0.15 M sodium chloride. Solutions previously used, such as borate buffer,[5] alkaline physiological saline,[4] Coca's solution,[7] phosphate buffer,[14] and buffered sodium desoxycholate did not increase the amount of isolated antigens or extract other complement fixing antigens. The use of 0.15 M saline, in addition to solubilizing the antigens, also expedited the adjustment of pH and ionic strength for the fractionation. Ethanol fractionation as described by Cohn *et al.*[20] at pH 6.0, ionic strength of 0.04 in respect to sodium chloride, and 16% ethanol, divided the 0.15 M saline extract into two distinct complement fixing antigens. The fractionated antigens were designated simply as ethanol soluble (ES) and ethanol insoluble (EI), and were purified further by physicochemical methods.

Purification processes involving the ES and EI antigens were performed in order to retain maximum serologic activity. Therefore, many methods of purification were attempted, including fractionation with ammonium sulfate, pH changes, varying pH and ethanol concentration, varying ionic strengths and ethanol concentrations, and cold, dilute trichloroacetic acid. Also, extractions with ethylene glycol,[30] chloroform,[31] phenol,[32] or pre-treatment with urea or guanidine[33] prior to ethanol precipitation were examined. In all these procedures, one or more serologically active fractions were obtained. However, there was usually a loss of serologic activity when compared to the initial total serologic activity. In addition, there was no evidence that these serologically active fractions were themselves distinct antigens. Frequently, it appeared that the serologic activity of a particular fraction resulted only because some of the studied antigens were coprecipitated.

The purest, most reactive ES antigen was isolated by virtue of its being precipitated by acetone and alcohol, heat stable, and insoluble at pH 4.6. The acetone and ethanol precipitations served a twofold purpose in that they brought about a purification of the antigen and simultaneously concentrated the material for the next step (Table 1). Following the acetone precipitation, the sediment was dissolved in a minimum volume of VBS (pH 7.4) and heated at 100°C to precipitate serologically inactive protein. To achieve maximum precipitation of the inactive material, the protein had to be concentrated, and at a pH between 7.2 and 8.0. Below pH 7.0 little purification by precipitation was realized; above pH 8.5, antigenic activity was destroyed.

The extraction of the ethanol precipitate with

acetate buffer pH 4.6 was performed three times, rapidly and in the cold. If additional extractions were employed, the antigenic activity of the ES antigen was reduced. This reduction in activity was attributed to the possible denaturation of the protein at the low pH 4.6, since the extracted material contained no antigenic activity in complement fixation tests. The material extracted at pH 4.6 contained considerable carbohydrate and formed a precipitation band with trichinella antisera in double diffusion system using agar gel.[21] It was noteworthy that following the removal of the carbohydrate the ES antigen was no longer stable to heating at 100°C. Moreover, other workers[34] reported this carbohydrate antigen, when freed from protein, was reactive in the skin test for trichinosis.

The EI antigen, following ethanol fractionation, was no longer soluble in 0.15 M sodium chloride. It was soluble, however, in a solution of sodium desoxycholate of pH 8.0 and high ionic strength. The sodium desoxycholate, while not necessary for solubilizing the EI antigen, allowed the elimination of undesirable lipoid material which was precipitable with calcium. The lipids, soluble in chloroform-methanol, exhibited no complement fixing activity with trichinella antisera.

The antigens could not be standardized on a dry weight basis because of the lability of the EI antigen to lyophilization. Therefore, undiluted antigen was considered as that amount of antigen isolated from 10 mg of dried larvae contained in 1 ml of solution. The EI antigen, although labile during lyophilizing, retained full antigenic activity for over 9 months when stored in 0.15 M sodium chloride at 3 to 6°C. The ES antigen, in contrast to the EI antigen, possessed maximum serologic activity even after lyophilizing or freezing, thereby demonstrating differences in the lability of the structural unit responsible for antigenic activity.

The methods employed in isolating an antigen frequently governs the composition, and hence, the reactivity of the antigenic components. Antigen prepared by the method of Witebsky *et al.*[4] has been shown to contain both the ES and EI antigens (Table 3). In our experience, however, the activity of different isolates of the antigen of Witebsky *et al.*[4] varied to a considerable extent. This could be explained by lability of the EI antigen to lyophilization, and also by coprecipitation of this antigen during the removal of serologically inactive protein by heat. Melcher[5] separated a borate buffer extract of dried larvae into two fractions by adjusting the pH of the solution to 4.8. The serologic activity of the acid soluble and acid insoluble fractions was determined in precipitin tests, and the acid soluble portion was reported as the active antigen. In our complement fixation studies, however, the acid soluble and acid insoluble fractions were found to have about the same antigenic activity; they could not be differentiated by complement fixation and could not be related to the ES and EI antigens. The seven serologically active fractions isolated by Labzoffsky *et al.*[6] also could not be related by us to the ES and EI antigens. Similarities exist, such as the early reactivity of fraction V of Labzoffsky *et al.* and the EI antigen, but rigid comparisons in respect to structure, ultraviolet absorption, and serologic activity were not possible.

The individuality of the ES and EI antigens was demonstrated by comparison of their ultraviolet absorption spectra (Figure 2), in serologic tests (Table 3), and by adsorption studies (Table 4). Marked differences were noted in each case. The chemical structures of the antigens, which were also quite different, will be discussed in another paper.

Both the ES and EI antigens were valuable in the diagnosis of trichinosis. This was shown by their reactivity with all sera from documented cases of human trichinosis and animals experimentally infected with *T. spiralis*. However, as was shown in Tables 5 and 6, the antigens reacted quite differently with trichinella antisera in respect to both time of reactivity and specificity. The earlier detection of antibodies to the EI antigen may result from the increased production of these antibodies or the greater sensitivity of the EI antigen. Antibodies reactive with the EI antigen could be responsible then for both reported early reactivity[6-8] and continued reactivity of sera from trichinella infections after several years.[10]

The ES antigen proved to be more specific in the diagnosis of trichinosis (Table 7). Only 4 weak reactions were recorded with 200 non-trichinella sera tested. The demonstrable greater specificity of the ES antigen, when compared with the antigen of Witebsky *et al.*,[4] resulted from the chemical fractionation and purification of the

former. The EI antigen, on the other hand, gave 23 reactions and 19 weak reactions with the studied sera. Cross-reactions were found with sera of patients with syphilis, schistosomiasis, hookworm filariasis, and systemic lupus erythematosus. The possibility of previous exposure to trichinella infections in these persons being responsible for most of these reactions is unlikely in view of the relative lack of reactivity of the healthy sera. Moreover, cross-reactions have been reported between sera from persons with trichinosis and an antigen isolated from *S. mansoni* cercaria.[35] Cross-reactions with sera from trichuriasis and ascariasis, which have been reported in the literature[12-14] were not found. Sera from *Dipylidum caninium* infections, also reported to cross react with trichinella antigens, were not available for testing.[11]

Despite its lack of specificity, the EI antigen might be profitably utilized in the diagnosis of trichinosis. In the first place, only 4 reactions were found with healthy sera. Secondly, the EI antigen was considerably more sensitive than the ES antigen and superior to the antigen of Witebsky *et al.*[4] in detecting early trichinosis infection. Therefore, the EI antigen could be used for screening suspected trichinella infections; then if reactive, later serum specimens might be tested with the ES antigen for confirmation of the diagnosis of trichinosis. The testing of sera with both antigens would have the dual benefits of sensitivity and specificity. The cross-reactivity of the EI antigen with sera of patients with schistosomiasis may be of little consequence since these infections are not endemic to the same areas, except with certain population groups which may have migrated from schistosome endemic areas. Cross-reactions with syphilitic sera would be detected by performing simultaneous serologic tests for treponemal antibody.[36] Positive tests with both the EI and ES antigens would be significant in establishing a serological diagnosis of trichinosis.

The lack of species specificity of the EI antigen may have possibly resulted from its deoxyribonucleoprotein structure. It has been shown that nucleoproteins react in complement fixation tests with "autoimmune" antibodies present in patients with systemic lupus erythematosus and other diseases.[37, 38] The EI antigen gave 2 reactions and 2 weak reactions with 5 sera from patients with systemic lupus erythematosus, which contained tissue antibodies.

Cross-reactions previously reported between trichinella antigens and typhoid antisera[15-17] were not demonstrated in complement fixation tests in this study. Whether the cross-reactivity between trichinella and typhoid is not demonstrable by complement fixation or whether the relation is essentially non-reciprocal, at least as far as the purified ES antigen is concerned, will require further study. However, crossreactivity was found between typhoid antigens and human trichinella antisera in agglutination tests. These tests revealed that some, but not all, human trichinella antisera will react with one or more of the *S. typhosa* antigens, O, H, and Vi. These results are in agreement with those found by Tompkins and Rogers[15] in agglutination tests using live bacteria. The failure of sera from *T. spiralis* infected guinea pigs to react with the typhoid antigen can, at this time, be attributed only to the peculiar inability of the guinea pig to produce a cross-reactive trichinella antibody. Weiner and Price[16] found that *S. typhosa* O and H antigens reacted with rabbit trichinella antisera, and later Weiner[17] reported that *S. typhosa* O antigens IX, XII_2, and XII_3 were the major components responsible for these heterogenic reactions.

SUMMARY

A method for the isolation and purification of two distinct complement fixing antigens from dried *Trichinella spiralis* was described. It consisted of an initial extraction with 0.15 M sodium chloride followed by fractionation with ethanol under conditions of controlled pH and ionic strength. Each antigen was purified further by physicochemical methods. The purified antigens, ethanol soluble (ES) and ethanol insoluble (EI), were shown to differ by their ultra-violet absorption spectra, in serologic tests, and by absorption studies.

The ES and EI antigens were evaluated in complement fixation tests against sera from both trichinella and non-trichinella infections. The ES antigen was shown to be quite specific for trichinosis. The EI antigen, on the other hand, lacked specificity, but proved to be extremely sensitive for the detection of trichinosis. The use of both antigens was suggested for the diagnosis of trichinosis in complement fixation tests. Evi-

dence for cross-reactivity between trichinella antisera and typhoid antigens was shown with agglutination reactions.

ACKNOWLEDGMENTS

The authors are indebted to Capt. James C. Burke, MSC, 406th Medical General Laboratory, Japan, and to Dr. Irving G. Kagan and Miss Lois Norman, Communicable Disease Center, Public Health Service, Atlanta, Georgia, for their generous supply of sera from persons with parasitic infections.

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STUDIES ON COMPLEMENT FIXING ANTIGENS ISOLATED FROM *TRICHINELLA SPIRALIS* LARVAE

II. Chemical Analysis*

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The chemical structure of trichinella larval antigens had been based previously on the study of relatively crude preparations. Earlier investigators had suggested a carbohydrate or glycoprotein structure for the active antigen. Bachman[1] stated that the trichinella antigen was undoubtedly a glycoprotein which was linked to a lipid. Taliaferro[2] also thought the trichina antigen to be a glycoprotein, but that the antigen was not readily soluble until the carbohydrate was removed by acid hydrolysis. Melcher and Campbell[3] isolated a polysaccharide which was reactive in intradermal tests. This polysaccharide antigen, however, was not reactive in complement fixation tests.[4, 5] Melcher[6] also prepared an acid soluble antigen which contained both protein and carbohydrate. Based on their solubility, Frisch *et al.*[4] believed the antigens isolated by Melcher,[6] and by Witebsky *et al.*[7] to be a carbohydrate, but different from the polysaccharide isolated by Melcher and Campbell. Labzoffsky *et al.*[8] prepared seven fractions from larvae which were active in complement fixation tests. Some fractions differed in their absorption of ultra-violet light, all fractions contained carbohydrate (Molisch test), and all fractions except one contained amino acids (ninhydrin) or protein (Biuret test).

The purification of two complement fixing antigens, isolated from *Trichinella spiralis* larvae, was described in the preceding paper.[8] The ethanol soluble (ES) antigen was specific for the diagnosis of trichinosis, but reacted later in the infection; while the ethanol insoluble (EI) antigen was not specific, but reacted earlier in the infection and antibodies to it persisted for a much longer time. The purification of these antigens provided an excellent opportunity to study their chemical structure. Therefore, chemical analyses and enzymatic studies were performed on the purified antigens in an attempt to determine the structure responsible for their antigenic activity in complement fixation tests.

* This paper is taken from a dissertation submitted to the Graduate School of Georgetown University in partial fulfillment of the requirements of the Ph.D. degree.

MATERIALS AND METHODS

Nitrogen was determined by a micro-modification of the method of Hiller *et al.*,[9] protein as described by Lowry *et al.*[10] using bovine serum albumin as a standard, carbohydrate by the method of Shetlar and Masters[11] using glucose as a standard, and phosphorus as described by Sperry.[12] Qualitative tests for pentoses, ketohexoses and galactose were performed as described in *Practical Physiological Chemistry*.[13] The anthrone method[14] was employed for hexoses, the colorimetric method of Elson and Morgan[15] for glucosamine and chondrosamine, and the method of Reissig *et al.*[16] for acetylglucosamine. Paper chromatograms for the detections of sugars were prepared as described in publication no. 5109 from the University of Texas,[17] and for basic constituents of nucleic acids by the method of Wyatt.[18] Glucose was determined using the glucose oxidase method of Saifer and Gerstenfeld[19] with "glucostat" reagent produced by Worthington Biochemical Corporation. Diphenylamine test for deoxyribose and the orcinol test for pentoses were performed as described by Dische.[20] Calf thymus deoxyribonucleic acid, a product of Sigma Chemical Company, was used as a standard. Lipids, after being extracted into chloroform-methanol (4:1) from an acid hydrolyzed solution, were spotted on filter paper and stained with Oil Red O.[21] Calcium was determined by the method of Horner.[22]

Ultra-violet absorption spectra were obtained with the Beckman DU spectrophotometer.

The reactivity of the antigens was studied in complement fixation tests after being subjected to the action of selected enzymes. The ethanol

soluble antigen was subjected to the action of trypsin (Pentex Corp.) and amylase (Nutritional Biochemical Co.), while the ethanol insoluble antigen was subjected to the action of trypsin and deoxyribonuclease (Worthington Biochemical Co.). Trypsin (0.5 mg/ml) was added to the antigens in veronal buffer pH 7.4. The reaction was allowed to proceed at 37°C for 4 or 20 hours, and terminated by the addition of an equal weight of soy-bean trypsin inhibitor (Pentex Corp.). Amylase activity was studied at pH 7.4, and test solutions were incubated for 1, 2 or 3 hours at 37°C. Deoxyribonuclease (0.5 mg/ml) was added to the ethanol insoluble antigen in veronal buffer pH 7.4 containing 0.1 M $MgSO_4$. The solution was incubated for 4 or 20 hours at 37°C, and the reaction followed by the increase in optical density at 260 mμ.

The method used for the complement fixation test was described in the preceding paper.[5]

TABLE 1

Quantitative chemical analysis of the ethanol soluble (ES) and ethanol insoluble (EI) antigens isolated from Trichinella spiralis *larvae*

Antigen	Per cent by Weight*			
	Protein	Carbohydrate†	Phosphorus	Nitrogen‡
ES	71–79§ 69–78‖	13–17	0.6–1.0	11–12.5
EI	13–15§ 41–49‖	less than 1	3.8–4.3	6.5–7.8

* Range of values from four different antigen extractions.

† Reported as glucose.[11]

‡ Method of Kjeldahl.

§ Method of Lowry *et al.*[10]

‖ Kjeldahl nitrogen multiplied by the factor 6.25.

RESULTS

The ethanol soluble (ES) and ethanol insoluble (EI) antigens were dialyzed against distilled water and then analyzed by qualitative and quantitative chemical methods. Preliminary studies revealed the ES antigen to contain protein, carbohydrate, but no detectable lipid. The EI antigen gave positive tests for protein, revealed small amounts of carbohydrate, and also contained no detectable lipid. As previously shown, the ultra-violet absorption spectra of the antigens were dissimilar.[5] The ES antigen had no distinctive absorption peaks, while the EI antigen gave maximum and minimum absorption at wavelengths similar to those observed with calf thymus deoxyribonucleic acid.[22]

Results of quantitative analyses on the antigens for protein, carbohydrate, phosphorus, and nitrogen are summarized in Table 1. The ES antigen contained, on the average, 75% protein and 15% carbohydrate. The nitrogen content was all in the form of protein as shown by the agreement between the protein values based on the nitrogen analyses and those found by the method of Lowry *et al.*[10] The antigen weight not accounted for by protein and carbohydrate was attributed to bound inorganic substances and water. On the basis of these data, the ES antigen was assigned a glycoprotein structure.

The EI antigen, on the other hand, contained only 14% protein, but a higher phosphate content. The carbohydrate value was low because the deoxyribose component was not reactive under the highly acidic conditions of the test.[11] The protein value, based on nitrogen analyses was noticeably higher than the value found by the method of Lowry *et al.*[10] The nonprotein nitrogen source was considered to be nucleic acid based on the ultra-violet absorption spectra and phosphorus content. The experimental data thus suggested a nucleoprotein structure for the EI antigen.

Additional tests were performed to study further the carbohydrate portion of the ES antigen and the nucleic acid portion of the EI antigen. Acid hydrolysates of the ES antigen, evaporated under vacuum and redissolved in water, were tested to determine the sugar moeity. This solution gave positive tests for hexoses with anthrone,[14] but tests were negative for ketohexoses,[13] pentoses,[13] and galactose.[13] Negative tests were obtained also for N-acetylglucosamine,[16] glucosamine, and chondrosamine.[15] Chromatographs of the hydrolysate on paper (Whatman no. 1) using butanol, acetic acid and water (4:1:1) as the solvent, revealed only a single brown band when sprayed with aniline phthalate. The Rf of the compound was that expected for hexoses, glucose, mannose, or galactose. Test sprays specific for pentoses and deoxysugars were negative.[17] The sugar was shown to be glucose by its reactivity in the glucose oxidase system.[19] Carbohydrate analysis

on an acid hydrolyzed sample of ES antigen by the specific glucose oxidase method[19] and the general carbohydrate method employing thymol,[11] gave values of 12.0 and 12.3% respectively. The agreement between these methods, plus the inability to find other sugars, showed that the polysaccharide of the ES antigen was composed entirely of glucose units.

The nucleic acid portion of the EI antigen was studied by analyses of the sugar group, and by paper chromatography of an acid hydrolysate. The sugar constituents were quantitated by the diphenylamine test for deoxyribose, and the orcinol test for pentoses. In the diphenylamine test, 850 μg of EI antigen yielded 520 μg of deoxyribonucleic acid, or 61% by weight. The orcinol test using 200 μg of EI antigen gave 131 μg of nucleic acid or 65% by weight. The agreement between the two assays showed that the nucleic content of the EI antigen was predominately of the deoxyribose form. Estimation of the nucleic acid content from ultra-violet absorption measurement at 260 mμ and 280 mμ gave a value of 55% nucleic acid, thereby, showing good agreement among the three different methods of nucleic acid determination. The nucleic acid content of the antigen was established, therefore, at about 60%.

The sum of the weight of the nucleic acid and protein accounted for about 75% of the weight of the EI antigen. The remainder of the weight was attributed to water, which according to Chargaff,[23] may account for 10 to 12% of the weight of nucleic acids and inorganic cations. The residual calcium content of several isolations was 1.4 to 2.0% of the lyophilized weight. No other group or groups of compounds were found during extensive qualitative analyses.

TABLE 2

Effect of trypsin and amylase on the antigenic activity of the ethanol soluble antigen

Enzyme	Reactivity* of antigen dil'd 1:						Antigen control
	5	10	20	40	80	160	
None	4	4	4	4	4	1	—
Trypsin	4	4	3	—	—	—	—
Amylase	4	4	4	4	1	—	—

* The numerical values represent the degree of complement fixation; 4, complete fixation; the negative sign (—), no fixation.

The basic constituents of the nucleic acid were released by acid hydrolysis (3 N HCl for 3 hours at 100°C). The hydrolysate was evaporated to dryness under vacuum, and the resulting residue dissolved in water. The solution was chromatographed on paper (Whatman no. 1) using as solvents butanol, acetic acid, and water (4:1:1), or butanol and water (86:14). The chromatograms, when examined with ultra-violet light (253.7 mμ), revealed 4 absorbing areas. These areas also reduced ammoniacal silver nitrate and formed sulfides with mercuric complexes. The ultra-violet absorbing areas of some chromatograms were marked, cut from the paper, and eluted overnight with 0.1 N HCl. The absorption spectra of the eluates were determined between 220 and 320 mμ. The curves which resulted from a plot of optical density against wavelength were identified as those given by the purines, guanine and adenine, and partially hydrolyzed pyrimidines, thymidine and cytosine.[24] The results revealed that the basic constituents of this deoxyribonucleic acid to be the same as those found from the thymus of calf, ox, sheep, and man.

The antigens were subjected also to the action of selected enzymes to study further the relationship between structure and antigenic activity. Table 2 summarizes the results found when the ES antigen was treated with trypsin and alpha amylase. Tests were conducted using a constant amount of trichinella antiserum. It was noteworthy that the antigen-enzyme controls were negative. Trypsin in 4 hours at 37°C reduced the antigenic activity of the ES antigen about 8-fold. This showed that the intact protein molecule was necessary for maximum antigenic activity. The use of soybean trypsin inhibitor had no observable effect on the complement fixation test. Amylase (0.5 mg or 1.0 mg/ml), when incubated with the ES antigen for 1, 2 or 3 hours resulted in a consistent, but relatively slight, loss of antigenic activity. These results could be interpreted to indicate that the carbohydrate portion of the antigen has only a minimum effect on the serologic activity. Moreover, the addition of exogenous glucose polymers, glycogen or dextran in amounts up to 5 mg per ml did not inhibit the reactivity of the antigen.

The antigenic activity of the EI antigen, on the other hand, was not affected by either trypsin or deoxyribonuclease when incubated at 37°C

for periods up to 20 hours. Also, the addition of calf thymus deoxyribonucleic acid did not inhibit the serologic activity of the EI antigen in complement fixation tests.

DISCUSSION

The ES antigen was assigned a glycoprotein structure with its carbohydrate portion composed of only glucose units. The carbohydrate moeity could be a glycogen molecule, based on the report of Flury,[25] who found large amounts of glycogen in *T. spiralis*. This glycogen possibly differed from the storage glycogen of mammals in that it was bound tightly to protein. Kent[26] reported glycogen was bound tightly to protein in the cestode *Hymenolepis diminuta*, and that it could not be removed without denaturing the protein. Attempts to separate further the protein and carbohydrate portion of the antigen by the chloroform gel method of Sevag *et al.*[27] resulted in a decrease in antigenic activity in complement fixation tests. Similarly, extraction with ethylene glycol to remove carbohydrate,[28] or phenol[29] to extract protein resulted in a decrease in serologic activity. This showed either that both the protein and carbohydrate portions of the antigen were necessary for serologic activity, or that the proteins were denatured during extraction processes and this was the cause of the observed decreased antigenic activity.

The role of the carbohydrate in the antigenic activity in complement fixation tests may be that of an auxiliary component. It has been reported, for example, that slightly antigenic molecules like casein or insulin become more antigenic when coupled to a carbohydrate.[30] The carbohydrate function in the glycoprotein complex of the ES antigen could be to furnish additional sites to attract and hold together the antigen-antibody complex. The slight decrease in antigenic activity obtained with alpha-amylase would tend to support this view.

The protein portion of the ES antigen molecule was deemed necessary for its activity in complement fixation tests. Heating a solution of the antigen at pH values above 8.5, where glycogen has been found to be stable, resulted in a considerable loss of antigenic activity.[5] This would indicate that the protein portion, or at least the protein-carbohydrate complex, was necessary for maximum serologic activity. In addition, the importance of the protein was shown when the antigenic activity was noticeably decreased by the action of the proteolytic enzyme trypsin. Wodehouse, using a phosphate buffer extract of larvae and rabbit antisera, studied the antigenic components by gel diffusion techniques. He found that treatment of the antigenic extract with trypsin removed half of the precipitating antigens. The fact that some antigenic activity remained after treatment with trypsin may be attributed to the antigenicity of the protein fragments formed by the action of the enzyme.

The EI antigen was analyzed and found to be a nucleoprotein with the nucleic acid portion being deoxyribonucleic acid. The low carbohydrate value that was found by the method of Shetlar and Masters,[11] resulted from the conversion of deoxyribose to levulinic acid under the acidic conditions of the test. Levulinic acid does not react with the thymol reagent.[11] The good agreement between the diphenylamine and orcinol tests showed the nucleic acid portion of the antigen to be predominately deoxyribonucleic acid. This was supported also by the identification of the basic constituents as adenine, guanine, thymine, and cytosine, which are normally found in many deoxyribonucleic acids.

The failure of trypsin to affect the antigenic activity of the nucleoprotein may result from the fact that the protein was tightly bound to the nucleic acid. Similar observations have been made with conjugated proteins.[31] On the other hand, the failure of desoxyribonuclease to effect the antigenic activity of the EI antigen indicated that the intact nucleic acid was not necessary for the antigenic activity. This is based on the assumption that the observed increase in optical density at 260 mμ resulted from the depolymerization of the nucleic acid by deoxyribonuclease. The experimental evidence was insufficient to affirm or negate either the protein or nucleic acid fragments as the antigenically active component in complement fixation tests. Since proteins generally are excellent antigens, however, and nucleic acids of dubious antigenicity,[32] it is most likely that the former constitutes the active antigenic substance of the EI antigen.

SUMMARY

The chemical structure of the ethanol soluble (ES) and ethanol insoluble (EI) antigens isolated from *Trichinella spiralis* larvae was determined. The ES antigen was found to be a glycoprotein

averaging 75% protein and 15% carbohydrate. The polysaccharide consisted entirely of glucose units. The EI antigen, on the other hand, was found to be a nucleoprotein averaging 60% nucleic acid and 14% protein. The nucleic acid portion analyzed to be deoxyribonucleic acid. Studies on the enzymatic breakdown of the antigenic substances inferred that the protein portion of each antigen was needed for activity in complement fixation tests.

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MICROFILARIAL GRANULOMA OF THE SPLEEN

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Microfilariae are usually found in the peripheral blood and also in the blood vessels of several internal organs of patients infected with certain types of filaria worms. The pathological changes produced by mature *Wuchereria bancrofti* and *Brugia malayi* in lymph nodes with resulting lymphadenitis, lymphangitis and lymphedema of the extremities in the later stages, as well as creeping swelling caused by *Loa loa*, are well known. In contrast, the pathogenicity and the life history of the microfilariae have not been clearly determined. It is questionable that microfilaria can cause any lesion in the body. Manson[1] believed the healthy fully formed microfilariae in the blood were harmless. O'Conner,[2] however, indicated that microfilariae were destroyed in the substance of the lymph nodes and were responsible to some extent for the pathological changes in these structures. Bonne,[3] in 1939, described an infestation of the splenic tissue with microfilariae in an Indonesian male, killed in a motor accident, in which no gross lesion of the spleen was noted. In 1941, Dhayagude and Amin[4] reported 11 autopsies in which granulomata were observed in the spleen and microfilariae of *W. bancrofti* were demonstrated microscopically in the granulomata. They believed the microfilariae were destroyed in the spleen and were the causative agent of those lesions. In their series microfilariae were observed also in other internal organs which were free of granuloma. In 1945, van der Sar and Hartz[5] reported 4 cases of tropical eosinophilia. In one, microfilariae were definitely demonstrated in the axillary lymph nodes associated with exudation of eosinophils, and in another one, there were minute subcapsular splenic nodules containing eosinophils and microfilariae. They did not determine the species of the microfilariae but mentioned that only *W. bancrofti* had been found in that area (Curacao). In connection with *Loa loa*, Klotz[6] described two examples of fibrotic splenic nodules in West African patients who were infected with this filaria and in whom microfilariae were observed in the nodules as well as in the blood. He postulated that the condition of nodular splenic fibrosis originated from the slow growth of fibroblasts in response to irritation by microfilariae. Roper,[7] however, considered those fibrotic nodules were not caused by microfilariae and their presence in the nodules was coincidental. Recently Duke[8] reported his findings, based upon experimental study in monkeys infected with *Loa loa*, and demonstrated that granulomata of the spleen occurred in those animals as the result of destruction of microfilariae within the organ. It appears then that the microfilaria of *W. bancrofti* is capable of producing granulomatous inflammation in the spleen and probably in the lymph nodes of man, and that of *Loa loa* is capable of producing granuloma in the spleen of monkey.

The purpose of this communication is to present a report of a case, in which microfilarial granulomata were encountered, in order to discuss further the pathogenicity of microfilariae and the pathogenesis of the granuloma.

Case Report

A 33-year-old male, Chinese was found dead in a pawn shop in Bangkok. The body was delivered by the authorities to the medico-legal department of the Police Hospital for postmortem examination. It was known that at least one year prior to his death he had been living in Bangkok.

The autopsy was performed approximately 32 hours after death. Externally, no signs of trauma were found. Internal examination revealed petechiae of the pericardium, pleura and pelvis of the kidneys. The liver as well as the lungs was congested. The spleen was enlarged about double the normal size, but the weight was not recorded. On section of the spleen, multiple, discrete, circumscribed, spherical nodules measuring up to 5.0 cm in diameter were noted. Each nodule had a solid, dark brown and bulging cut surface. The unaffected splenic tissue was firm and dark purplish red. Other organs were not described. Blocks of tissue were taken from the spleen,

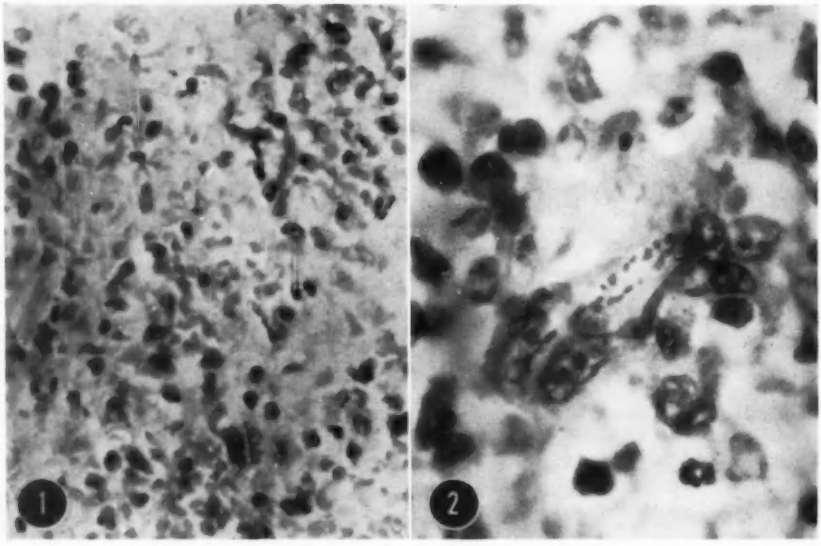

FIG. 1. Section of spleen showing eosinophils, histiocytes and mononuclear cells infiltrating loose fibrous connective tissue with hemorrhage in the splenic nodule. H & E × 450.

FIG. 2. Section of spleen demonstrating degenerated microfilaria surrounded by histiocytic cells. H & E × 1,000.

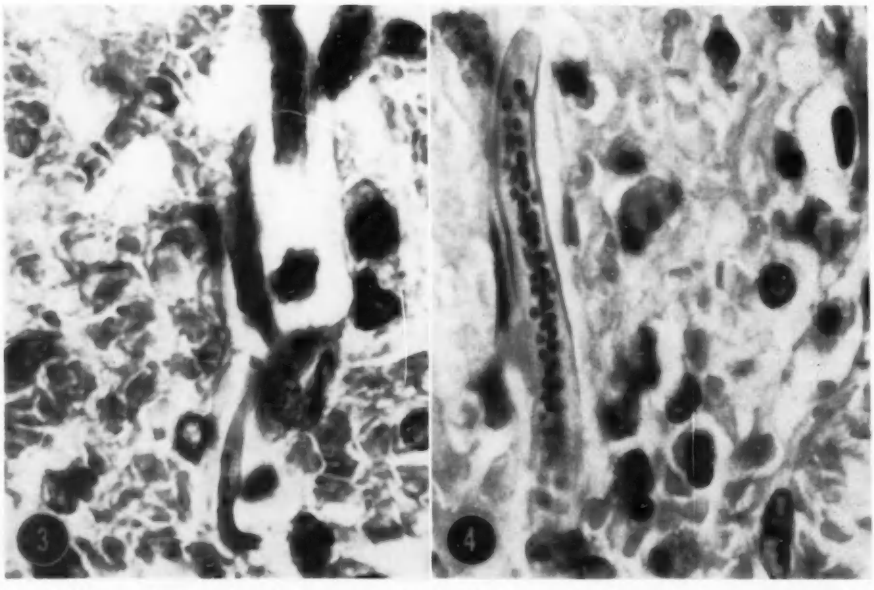

FIG. 3. Section of spleen showing segments of microfilaria partly adherent to the wall of a capillary. The segment at the bottom shows terminal nuclei in the tail. H & E × 1,200.

FIG. 4. Anterior end of a microfilaria in a spleen capillary showing the cephalic space and nuclei. H & E × 1,200.

lungs, liver and kidneys. The content of the stomach was submitted for toxicological study and salicylate compounds were found.

Microscopic examination of the spleen revealed all nodules to be similar in structural pattern. The nodules were sharply demarcated, but no fibrous capsule was present. Within the boundary of each nodule, there were many small veins, capillaries, arterioles and arteries supported by loosely arranged hyalinized fibrous connective tissue which became denser around the blood vessels. Interspersed and embedded in the connective tissue were numerous young fibroblastic cells, small and large mononuclear cells, plasma cells and a large number of eosinophilic leukocytes (Figure 1). Many aggregates of histiocytic cells resembling epithelioid cells surrounding degenerated microfilariae (Figure 2), as well as multinucleated giant cells, some of which contained microfilarial nuclear bodies, were noted in the lesion. Small petechial hemorrhages were frequently noted in the nodule. The remainder of the splenic tissue showed marked congestion of the red pulp with diminution of the white pulp and the malpighian corpuscles. A large number of segments of microfilariae were seen both inside and outside of the nodular blood vessels. Inside the blood vessels they were well stained and often were adherent to the wall of the blood vessels (Figures 3, 4). Microfilariae were not observed outside the nodules. Sections of the lungs revealed marked congestion, edema and rather diffuse intra-alveolar hemorrhage. The liver also showed marked congestion of the sinusoids and the presence of occasional small aggregates of eosinophils and mononuclear cells. The kidney demonstrated congestion of the blood vessels. Segments of microfilariae were also observed in the blood vessels of the liver and the kidney, but no tissue reaction similar to that in the spleen was noted.

DISCUSSION

It was obviously demonstrated in the case reported that the granulomatous lesion was present, as far as could be determined, only in the spleen. As to the etiology of the lesion, we are in agreement with Dhayagude and Amin[4] who concluded that lesions of this particular nature were microfilarial in origin. This conclusion is also supported by the work of Duke,[8] who produced splenic microfilarial granulomata experimentally in monkeys infected with *Loa loa*. The lesion, as seen in this case, was made up primarily of large histiocytic cells similar to epitheloid cells, mononuclear cells and eosinophils with a considerable amount of fibrous connective tissue formation situated in between the interstices of vascular channels. It should be emphasized here that segments of the microfilariae were observed both inside and outside of the vascular channels. Those located inside the vascular channels were apparently viable and healthy microfilariae but those outside were often degenerated and being engulfed by histiocytic cells which in some places were transformed into multinucleated giant cells containing the remains of the microfilariae. From this particular observation, it can be evidently assumed that the microfilariae are able to migrate out of the vascular channels at least in the spleen and when they are outside a tissue reaction, characterized as granulomatous inflammation with eosinophilic infiltration, occurs in order to destroy the foreign organisms. This assumption can also be used in explaining the finding that granulomata were not observed in other organs in which the microfilariae were present because they were all inside the vascular channels. In the lymph node of a case reported by van der Sar and Hartz,[5] it was observed that the microfilariae in the eosinophilic abscesses were probably dead and were different from those inside the lumen of blood vessels. However, it still can not be explained why the microfilariae were largely concentrated and migration should occur only in the spleen and, probably, in the lymph node. It is also interesting to note the enlargement of the spleen in this case as well as in cases reported by the others.[4, 5]

Hemorrhage which was present in the granulomata of the spleen in this reported case was considered to be a coincidental finding of the generalized hemorrhagic process which evidently resulted from a toxic substance and was not related to the migration of the microfilariae.

The rarity of the granuloma in the routine autopsies in areas where filariasis is prevalent such as in Thailand is of great interest. However, this should be anticipated in the light of the experimental studies reported by Duke.[8] He indicated that the microfilarial granuloma could undergo a process of resolution in monkeys when the microfilariae no longer invade the peripheral blood. In human cases of filariasis caused by

W. bancrofti and *B. malayi*, it is already well known that microfilariae often are observed in the peripheral blood only at the early stages of the disease.

The microfilaria in this case could not be identified positively, as none except those in tissue sections were available for examination. It could be established, however, that the microfilariae were sheathed, were of the same diameter and length as those of *W. bancrofti* when fixed after death and sectioned in tissue, and the arrangement of nuclei in both extremities of the body were identical with that most commonly seen in *W. bancrofti*.

SUMMARY

A case of splenic granulomata in a male Chinese in Thailand is described. The lesion is believed to be caused by microfilariae of *Wucheria bancrofti*. It is assumed that the granulomatous process occurred as the microfilariae migrated out of the vascular channels and were destroyed in the spleen. The low incidence of the observed lesion is probably due to the reversibility of the process.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Tawal Asanasen of the medico-legal department of the Police Hospital and Prof. Pirom Suvantemiya, head of the Department of Pathology, Chulalongkorn Hospital Medical School, for their permission to report the case.

Gratitude is also expressed to Prof. Paul C. Beaver, Tulane University School of Medicine, New Orleans, Louisiana, for his kind efforts in identification of the microfilaria.

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LYMPHANGIOGRAPHY IN FILARIAL LYMPHOEDEMA AND ELEPHANTIASIS*

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Non-filarial elephantiasis or lymphostatic verrucosis is not uncommon in East Africa. The clinical features have been described by Loewenthal[1] in Uganda and by Clark[2] in Kenya. Cohen[3] has reviewed the problem and has given a detailed description of the underlying pathology with special reference to lymphangiography. Filarial elephantiasis is much more prevalent but surprisingly little is known about the appearance of the lymphatics in this condition. We have in fact seen no reports of x-ray lymphograms having been made in subjects with filarial elephantiasis.

Technically there is no special problem in making x-ray lymphangiograms; the less severe cases show a common pattern with extensive varicosities which clearly differentiate them from the pattern in non-filarial lymphoedema in East Africa.

Selection of Cases

By inspection of the limb alone it is impossible to distinguish filarial from non-filarial elephantiasis. Both conditions may progress through the stage of pitting lymphoedema and recurrent lymphangitis to elephantiasis with thickened skin and warty excrescences.

It was essential to select cases from an area where *Wuchereria bancrofti* transmission was present. In Kenya bancroftian filariasis is rarely seen except along the coast where it is very common. Recent studies on the Island of Pate[4] and in 36 villages in the Coast Province (Nelson *et al.* in preparation) have confirmed the observations of Jordan[5-7] that in Tanganyika in areas of active transmission the elephantiasis and hydrocele rates are usually proportional to the *W. bancrofti* infection rate. At Faza on the Island of Pate the infection rate in males over the age of 15 years was 40%, the elephantiasis rate 16%, and the hydrocele rate 39%. In the Malindi area where cases were selected for lymphangiography, the infection rate in males over the age of 15 years was 28.3%, the elephantiasis rate 5.8%, and the hydrocele rate 34.5%.

In selecting cases of filariasis for lymphangiography, we ensured that they all came from villages where the *W. bancrofti* rate was high. All gave a history of filarial fever and they all had either typical filarial lymphoceles or hydroceles (Figure 1). Night blood slides in the selected patients were negative for microfilariae. This was not surprising as in a previous survey on the coast only 4 out of 65 subjects with elephantiasis of the leg had microfilariae in their blood, although the mean infection rate in the adult population in the endemic area was 35%. The low density of microfilariae in persons with undoubted filarial elephantiasis is well known and has been confirmed in East Africa by Heisch *et al.*[4] and Jordan.[7] Lymphoceles or "hanging groin" are a common feature of onchocerciasis in East Africa,[8] but it is doubtful if true elephantiasis is a complication of this infection. However, onchocerciasis does not occur on the Kenya coast. We have no doubt that the 8 patients selected for lymphangiography were all cases of lymphoedema or elephantiasis due to *W. bancrofti* infection.

Filarial elephantiasis of the leg goes through two clinical stages. Early in the course of the disease the affected limbs are swollen and there is pitting edema; this stage will be referred to below as filarial lymphoedema. Later in the disease the skin is thickened, warty and woody hard, and the leg is greatly enlarged. This is the stage of elephantiasis.

Of the 8 patients selected 6 had well defined filarial lymphoedema of the legs and three of these were bilateral. Two had elephantiasis. Seven had hydroceles and the remaining patient had a well defined lymphocele.

Lymphangiography

The technique used was that described by Kinmonth,[9] but it was found that 50% diodone was easier to inject. This had the slight disad-

* This project was financed by a grant from the African Research Foundation, Box 9202, Nairobi, Kenya.

† Director of the African Research Foundation.

FIG. 1. A patient showing filarial lymphoedema of the leg, bilateral hydroceles and a well defined left lymphocele.

vantage that the resultant x-rays were less well defined. The absence or the presence and character of lymphatic trunks can be readily demonstrated by making an incision over the dorsum of the foot after injecting 0.2 ml of patent blue violet into the webs of the toes. After a few minutes the dye enters the lymphatic vessels and this process is helped by moving the knee and ankle joints. This procedure of dissection and inspection is usually termed visual lymphangiography. If the lymphatic trunks are patent they fill with dye and it is possible to inject them with a radio-opaque fluid such as diodone. A Mantoux needle on a 10-ml syringe is used and the radio opaque material is injected as rapidly as possible. Pressure with a gauze swab over the needle in the lymph vessel controls leakage. An x-ray lymphogram can then be made after an interval of 2 minutes.

In the two legs with elephantiasis no lymphatics were found. In the patients with lymphoedema normal lymphatic trunks were seen in the foot. However, an incision at ankle level just lateral to the medial malleolus showed at least one obviously dilated lymphatic trunk in each case Exposure of enlarged femoral lymph nodes in two patients showed normal lymphatic vessels entering the nodes.

X-ray lymphangiography showed a common pattern in five legs with lymphoedema. In one patient no picture was made as the x-ray plant failed during the operation. The lymphograms showed dilatation and tortuosity of the lymphatic trunks with filling of the collecting radicals and dermal back flow. More lymphatic trunks were apparent than in a normal lymphogram and some of the abnormal trunks ended blindly (Figures 2 and 3). This latter observation was confirmed by visual lymphangiography in a patient undergoing a Charles' type of operation. The diseased lymphatic trunks were dissected proximally and some were found to end blindly in fibrous tissue.

An x-ray lymphogram made in 1959 in Mombasa of the lymphoedematous leg of a patient with the classical signs of filariasis showed the same changes of varicosity, filling of the collecting radicals and dermal back flow in the lower part of the leg (Figure 4).

DISCUSSION

Varicose lymphatic trunks have been demonstrated by x-ray lymphangiography in 6 patients with filarial lymphoedema of the legs. Although a proportion of lymphatic trunks are shown to end blindly, there are numerous anastamotic channels and there is no obstruction to the passage of the dye up the leg. The dilatation is presumably a local reaction to the presence of the adult worms, as in two patients the lymphatic vessels in the feet and groins were examined and found to be normal. In the clinically normal legs in two patients with unilateral lymphoedema, early changes in the form of localized segments of tortuosity can be seen in the x-ray lymphograms but there is no obstruction to the proximal flow

FIG. 2. Lymphogram in filarial lymphoedema
FIG. 3. Normal lymphogram

of dye through the abnormal segment (Figure 5). This would appear to correspond to Lichtenberg's[10] stage of lymphangiectasia. We have seen similar tortuous and varicose lymphatic vessels in Kenya dogs and cats infected with *Brugia patei*; and in these animals careful search has revealed the adult worms thrashing to and fro in the dilated lymphatic vessels.

This pattern of well developed varicosities in the lower part of the leg has only been seen by us in patients with filariasis. However, Kinmonth (personal communication) has seen similar pictures in non-filarial types in England and Wallace *et al.*[11] have published a similar lymphangiogram made in a man with traumatic lymphoedema in America.

Even in early cases of lymphoedema repeated night blood slides failed to reveal microfilariae although it was demonstrated that there was no sealing off of the lymphatic systems of the affected legs. It is evident that mechanical obstruction is not responsible for the absence of microfilariae from the blood. Similar behavior is seen with other filarial species where normal, fertile adult female worms are found but microfilariae are absent from the blood.

Enlargement of the regional lymph nodes bore no relationship to edema of the leg and this sug-

FIG. 4. Lymphogram in filarial lymphoedema in a patient from Mombasa

gests that the lymph nodes are not the site of obstruction to the system (Figure 1). Total blockage of lymphatic trunks in a limb causes atrophy rather than dilation of the distal lymphatics. In a patient with non-filarial elephantiasis of the leg and thigh following block dissection of the lymph nodes in the groin for malignant melanoma, no lymphatic vessels could be demonstrated after careful dissection with injections of patent blue violet at various levels. A similar result was obtained by excising sections of the main lymphatic trunks in the legs of two *Cercopithecus mitis* monkeys. After an interval of 8 weeks the lymphatic trunks were not dilated and

FIG. 5. Early changes in the apparently unaffected leg of a patient with filariasis

FIG. 6. Lymphogram in lymphostatis verrucosis showing solitary hypoplasia (from *East African M. J.*, **37** (no. 2): 53).

in one they were appreciably smaller than they had been previously.

These findings suggest that the original lesion in the lymphatic trunk may be a localized one brought about by the presence of the adult worms, as suggested by Lichtenberg.[10] The valvular function of the lymphatic trunk is destroyed and back flow and stagnation result. The lymph soaked limb is now susceptible to infection by non-specific organisms and the way of life of these patients encourages such infections. The end result, as in lymphostatic verrucosis, is the destruction of both the lymphatic trunks and the superficial lymphatic plexuses leading to elephantiasis[3]. O'Connor[12] clearly demonstrated that when the parasites die they set up foreign body reactions which block the lymphatic trunks. Our observations suggest that this may be a late result

and not the cause of the lymphoedema as is generally supposed. Lymphograms show that lymphoedema is well established even when injection of dye shows no obstruction to the flow of lymph up the leg.

In East Africa, as elsewhere in most endemic areas, there is an increase in the incidence of lymphoedema with increase in the transmission of *W. bancrofti* irrespective of race or tribe. There seems to be little doubt that the severity of the lesion is proportional to the worm load.

Three types of lymphoedema are common in Kenya: oedema associated with phlebitis, lymphostatic verrucosis, and filarial lymphoedema. Edema due to phlebitis has been described by Manson-Bahr.[13] The edema is restricted to the leg and the foot is unaffected. A venous block can be demonstrated by a phlebogram and the lymphogram is normal.

In lymphostatic verrucosis destruction of the lymphatic system of the leg takes place early in the course of the disease, but in very early cases an underlying hypoplasia of the lymphatic trunks can be demonstrated by lymphangiography (Figure 6).

In patients with filarial lymphoedema the lymphograms are markedly different from those in lymphostatic verrucosis. Varicose lymphatic trunks are demonstrated in these patients with a degree of lymphoedema and a length of history which in lymphostatic verrucosis would be associated with destruction of the lymphatic trunks. In filarial disease the lymphatic system may eventually be destroyed and elephantiasis results. Thus, in both diseases the final result is the same although the mechanism is different. In lymphostatic verrucosis a hypoplastic lymphatic system is destroyed early in the course of the disease by lymphangitis. In filariasis a normal lymphatic system is invaded by parasites. If only a small segment of one or two lymphatic trunks is affected there is no lymphoedema. If several or all of the lymphatic trunks in the leg are involved in the course of repeated filarial infections, the system becomes so incompetent that lymphoedema results. Finally, non-specific lymphangitis combined with the death of the adult worms due to senescence or immunity destroys the system and leads to irreversible elephantiasis.

SUMMARY

Nine patients with filarial lymphoedema or elephantiasis were investigated by lymphangiography. In Kenya x-ray lymphangiograms made in patients with filarial lymphoedema show a typical pattern of varicosities which clearly differentiate this condition from lymphostatic verrucosis, the prevalent form of non-filarial lymphoedema. Microfilariae are absent from the blood even when there is no obstruction to the flow of lymph in the affected leg. In advanced cases there is total obliteration of the lymphatic system of the leg.

ACKNOWLEDGMENTS

We are grateful to the Permanent Secretary/Chief Medical Officer of the Ministry of Health and Welfare for providing facilities at Malindi Hospital. We are also indebted to Dr. Z. Rossinger, Dr. G. Kelham and Mr. G. O'Ketch for technical assistance.

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THE EFFECT OF PREDNISONE ON PERSISTENT MICROFILAREMIA DURING TREATMENT WITH DIETHYLCARBAMAZINE

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It was reported by Hawking[1] in 1950 that a small percentage of microfilariae of the periodic type of *Wuchereria bancrofti* persists in the peripheral blood during treatment with diethylcarbamazine (Hetrazan®, Banocide®) in full dosage. Otto *et al.*[2] noted the same phenomenon when treating infections of the non-periodic type and also found that a second course of treatment three months later did not kill as high a proportion of the circulating microfilariae as the first course had. Hawking said that this persistent microfilaremia stays constant during treatment and that it is not due to inadequate dosage of the drug. He considered that it was not due to a failure of the drug to sterilize the adult female worms because, if this were the explanation, a steady increase in microfilarial counts should start from the time treatment was discontinued and this does not occur.

Diethylcarbamazine given by mouth was found by Hawking[1] to remove most microfilariae from the peripheral blood in vivo but not from filarial hydroceles, although the drug reached hydrocele fluid in high concentrations. It did not produce any visible effect on live microfilariae added in vitro to the serum of a patient taking the drug. Hawking *et al.*[3] showed that the microfilariae of *Litomosoides carinii* in cotton rats treated with diethylcarbamazine are finally destroyed by macrophage cells in the liver, and Woodruff[4] demonstrated that this also applied to the microfilariae of *Loa loa* in man.

Hawking[1] found that most *W. bancrofti* embryos disappear from the peripheral blood within a few hours of the drug's first administration by mouth. He suggested that the mode of action of diethylcarbamazine could be likened to that of an opsonin because, in some way, it appears to condition the microfilariae to quick destruction by the body's defenses to which they do not otherwise rapidly succumb. Murgatroyd,[5] during the discussion of Hawking's paper, suggested that diethylcarbamazine may potentiate some microfilaricidal substance produced by the patient. Consequent upon Murgatroyd's suggestion we thought that the corticosteroid compounds, which can interfere with some antigen-antibody reactions, might be found to lessen the drug's microfilaricidal effect in vivo.

The corticosteroids are used sometimes to control severe allergic reactions which patients under treatment with diethylcarbamazine may develop as a result of the liberation of filarial protein. Adrenocorticotrophic hormone (ACTH) also was used successfully by Thompson[6] to control reactions in a case of loaiasis. McFadzean[7] used quite small doses of ACTH to treat bancroftian elephantiasis but found it had no apparent effect on the edema. Markell and Kerrest[8] found that cortisone lessened limb edema in elephantiasis. They also reported one case of elephantiasis in which a very gradual rise in mean microfilarial counts occurred after 20 days of a course of cortisone, with a fall when the steroid was discontinued. They suggested that the slow rise in this case was due to the cortisone having produced a reduction in the inflammatory blockage of the lymphatics, thus allowing more microfilariae to reach the blood. Jordan[9] noted a similar occurrence when giving a course of prednisolone to a patient with elephantiasis. In his case, also, the increase in microfilarial counts did not become apparent until treatment had been continued for 3 weeks. He suggested that in such cases the corticosteroids might have interfered with some immune process.

The purpose of this investigation was to determine whether cortiocosteroids would increase the number of microfilariae that persist in the peripheral blood during diethylcarbamazine treatment. To this end a group of patients treated with prednisone plus diethylcarbamazine (group I) have been compared with a group, as similar as possible in all other respects, treated with diethylcarbamazine alone (group II). The findings of Markell, Kerrest and Jordan outlined above suggest that the delayed increase in microfilarial counts that occurs with administration of the steroid alone would not complicate

the picture if the experiment were completed well within 20 days.

MATERIALS AND METHODS

The patients studied. For the main experiment healthy people without any disease that could be made worse by prednisone or ACTH were needed. High microfilarial counts were also desired. Such people are most likely to be found in villages in endemic areas. Race[10] had reported a microfilaremia rate of over 50% in people more than 14 years of age in the Cape Gloucester district of New Britain so these people were chosen. Young adult villagers who lived close to the small hospital at Hill 660, Borgen Bay, agreed to be treated. The patients slept, were fed and given the drugs 6-hourly at the hospital but otherwise led their ordinary lives. Nobody with less than 30 microfilariae in 20 cmm of night blood was accepted in order that in all cases, during treatment with diethylcarbamazine, there would be enough persisting microfilariae to allow some to be found each night in 20 cmm of blood. A full physical examination was made and only people with no clinical signs of filariasis or any other disease, except malarial splenomegaly, were treated.

Blood examination. Twenty cmm of blood was taken by finger prick every night from each patient by the same individual at the same time, between 10 and 11 p.m. The blood was spread evenly on a slide and immediately examined under low power for visible microfilariae. The blood then dried overnight and was dehemoglobinized in the morning. After being allowed to dry again, it was fixed in methyl alcohol and stained with Giemsa. All the microfilariae present were then counted at our convenience.

The main experiment. On the 1st night after the blood had been taken, group II's prednisone, in the dosage described below, was started at midnight. On the 2nd night the course of diethylcarbamazine was started in both groups at midnight. The 3rd night's microfilarial counts were disregarded because Hawking[1] found that diethylcarbamazine by mouth does not exert its optimal microfilaricidal effect for 48 hours. The 4th, 5th, 6th and 7th nights' counts in group I were then compared with those of the same nights in group II.

At midnight on the 7th night the prednisone given to group I began to be reduced in dosage by steps to zero over 36 hours, *i.e.*, the last full dose had been given at 6 p.m. on the evening before the 7th night and none was given at 6 a.m. on the morning after the 8th night. The 8th night's counts were disregarded. The mean of the 9th and 10th nights' counts in group II were then compared with that of the 4th to 7th nights in the same group, on the supposition that much of the prednisone's effect would have disappeared in the intervening period. The diethylcarbamazine was discontinued in both groups at midnight on the 10th night.

ACTH. Two men, instead of receiving prednisone, were treated with quick-acting ACTH for 6 days which was then reduced by steps to zero. They were given diethylcarbamazine in the same dosage as the other patients.

Prednisone alone. Previously, at Port Moresby Hospital, two men had already been given only prednisone for the same length of time and in the same dosage as group I, to establish whether the corticosteroid alone might alter microfilarial counts over the length of time for which group I later received the drug. Blood slides were made from them at midday, also, in order to make sure that prednisone did not alter the nocturnal periodicity of the microfilariae.

Drug dosage. The corticosteroid used in this study was prednisone as it has less side-effects than cortisone. The dosage given, in all fifteen cases (group I) and to the two men in Port Moresby, was 0.6 mg per kg per day in four divided doses, at midnight, 6 a.m., noon and 6 p.m. It was thought unjustified to continue the prednisone at this dosage for more than 6 days lest the patients' adrenal functions became dangerously depressed. During the subsequent 36 hours the dosage was therefore reduced by steps to zero. The ACTH given to the other two men was in low dosage, 10 units twice daily.

The diethylcarbamazine was given in dosage of 6 mg per kg per day, also in four divided doses at the same times of the day as the prednisone. Although this drug is said to be stable under tropical conditions, care was taken to ensure that both groups of patients were given their tablets out of the same bottle. We weighed the patients and gave all the treatment ourselves.

Mathematical methods. To exclude the possibility that the degree of variation in microfilarial counts before treatment might have differed

TABLE 1

Effect on microfilarial counts of diethylcarbamazine plus prednisone (group I) compared with diethylcarbamazine alone (group II) (4th to 7th nights)

	Group I	Group II
No of cases	15	7
(a) Mean count before treatment	149.1	146.4
(b) Mean count persisting during treatment, as a percentage of (a)	4.30	1.34
(c) Range of counts during treatment, as percentages of individual counts before treatment	1.84–12.50	0.17–2.66

TABLE 2

Effect on microfilarial counts in group I of discontinuing prednisone but not diethylcarbamazine (4th to 7th nights compared with 9th to 10th)

	Prednisone and diethylcarbamazine	Diethylcarbamazine alone
No. of cases	15	15
Mean count persisting during treatment*	4.30	2.56
Range of counts during treatment, as percentages of individual counts before treatment	1.84–12.50	0–4.92

* As a percentage of (a), Table 1.

in groups I and II the value of V (variance) was calculated for each group. No difference in variance was found by the F test.

Although a considerable difference in the numbers of microfilariae in the blood of any untreated individual may often be found from one night to another this applies much less to treated patients. Hawking[1] found, in 60 cmm of blood, a range of between 1 and 4 microfilariae persisting during diethylcarbamazine treatment. However, as patients with high microfilarial counts before treatment might be expected to have higher counts during treatment than patients with lower original counts, it was thought that a comparison between groups I and II should be based upon the proportion of microfilariae that persisted in each case rather than upon a direct comparison of the whole numbers of persisting microfilariae. Therefore, the mean microfilarial count for each patient during the 4th to 7th nights inclusive was calculated and this was expressed as a percentage of the count found in his case before any treatment had been given. The means of these percentages in each group were then compared statistically. In the same way, within group I, the counts on the 4th to 7th nights were compared with those on the 9th and 10th nights. The methods of statistical analysis used are described by Swaroop.[11]

RESULTS

The effect of prednisone alone. In 15 cases (group I) the microfilarial counts on the 1st and 2nd nights were compared, *i.e.*, before and after 24 hours of prednisone alone. In 7 cases there was an increased count, in 7 cases a decrease, and in one case no change in count. The mean microfilarial count of the 15 cases remained virtually unchanged.

In the case of the two patients in Port Moresby the counts for 6 nights without treatment were compared with those for 6 subsequent nights during prednisone treatment. No significant difference in mean counts was found and the nocturnal periodicity was unaltered.

The main experiment. Results of the main experiment are summarized in Tables 1 and 2. The mean count of persisting microfilariae was higher in the group receiving diethylcarbamazine plus prednisone than in the group receiving diethylcarbamazine alone. The standard error of the difference between the mean percentages of persisting microfilariae in the two groups is 1.34, $t = 2.208$, $p < 0.05$.

After prednisone was discontinued, the mean microfilarial count was 2.56 as compared with the earlier mean of 4.30. The standard error of the difference between the two means is 0.84, $t = 2.078$, $p < 0.05$. In group II the mean persisting microfilarial count was the same on the 9th to 10th nights as it had been on the 4th to 7th.

The effect of ACTH. In one case 10% of microfilariae persisted during diethylcarbamazine plus ACTH treatment, compared with 2% after the ACTH had been discontinued. In the other case, however, only 1% persisted throughout.

The loss of some microfilariae during staining. Throughout treatment, either with diethylcarbamazine alone or with both drugs, persisting microfilariae were seen moving on direct examination. Usually larger numbers would be counted on each slide after the blood had been dehemoglobinized, fixed and stained next day. On two occasions, however, one more microfilaria was counted at night in a fresh preparation than was found after staining. Therefore, some microfilariae must sometimes be lost during preparation or staining and the technique used is not ideal. This probably need not affect the interpretation of the results of this experiment.

Complications of treatment. One man developed fever while taking prednisone. Falciparum malaria parasites were found in a thick film and the fever responded to chloroquine. After this, all the patients, including the control group, were given suppressive chloroquine. One man developed a myositis of the anterior axillary fold, while receiving prednisone and diethylcarbamazine, which responded to penicillin. Otherwise, no complications or sequelae of prednisone or ACTH treatment occurred.

In the control group, 3 out of the 7 patients developed fevers during the first 48 hours of diethylcarbamazine treatment, but these settled without reduction of the dosage becoming necessary. In the prednisone-treated group there were no diethylcarbamazine reactions except in the case of one man in whom tender enlarged inguinal and axillary lymphatic glands appeared without a rise of temperature. Presumably, adult worms had died at these sites. The condition settled without any alteration of treatment.

DISCUSSION

The results can be summarized as follows:

1. Prednisone given alone in usual therapeutic dosage had no discernible effect in 24 hours on the mean microfilarial count of a group of 15 patients with heavy asymptomatic *W. bancrofti* infections. In two other cases no effect on counts was noted during a 6-day course of prednisone alone and the nocturnal periodicity of their microfilariae was unaltered.

2. During treatment of a group of patients with diethylcarbamazine plus prednisone three to four times as many microfilariae persisted in the night blood as in a group treated with diethylcarbamazine alone. This difference in the mean counts of persisting microfilariae was statistically significant. In the first group, while the diethylcarbamazine was continued, the mean persisting microfilarial count had fallen to a statistically significant degree 48 hours after the administration of the prednisone began to be reduced and 24 hours after it had been stopped. Prednisone, therefore, produced an increase in persisting microfilaremia during diethylcarbamazine treatment.

3. The results of administering quick-acting ACTH instead of prednisone in two cases were inconclusive.

4. The technique employed to dehemoglobinize, fix and stain blood slides for microfilarial counts, which is that generally employed in the field, may sometimes result in the loss of some microfilariae.

The explanation of the findings summarized in paragraph 2 above is not clear because the cause of persistent microfilaremia during diethylcarbamazine treatment is still unknown. The possibility that the effect of prednisone was merely to interfere with non-specific mechanisms by which microfilariae are destroyed in the body at the end of their usual life-span is to some extent discounted by the finding, summarized in paragraph 1 above, that prednisone alone produced no observable increase in counts in two cases over 6 days, and none in the cases of Markell, Kerrest and Jordan in the first 20 days. On the evidence available, two theories seem possible—(A) the persisting microfilariae may come from adult worms in ectopic sites in the body, or (B) an antibody may be involved in the microfilaricidal action of the drug.

Hawking[1] suggested that persistent microfilaremia during treatment could be explained if there were a store of microfilariae in the body which were not killed by the drug, as he demonstrated there is in patients with hydroceles. Later Bertram[12] suggested, on epidemiological grounds and on analogy with his findings in cotton rats superinfected with *L. carinii*, that in heavily infected people the adult worms may have primary and secondary sites in the body. O'Connor,[13] in a review of the pathological evidence in man, showed that adult filarial worms have often been found, in states of hyperinfection, outside their usual sites. He believed that there was evidence that microfilariae from such worms can gain access to the venous blood without passing through the lymphatic system.

The fact that prednisone, as is stated in paragraph 1 above, did not produce a discernible increase in microfilaremia before diethylcarbamazine was given does not invalidate this hypothesis. A three to fourfold increase in the small number of microfilariae seeping in from these ectopic sites would not be detectable while the drug-susceptible majority still greatly outnumbered them in the peripheral blood. Such an increase in a small minority could only be expected to become apparent in the cases described here when the susceptible majority had been eliminated after the beginning of diethylcarbamazine treatment.

It may be that the prednisone, by decreasing tissue barriers to the passage of microfilariae, allowed more of them to filter into the peripheral blood from ectopic sites than would otherwise have been able.

The second theory depends upon the possibility that filarial patients produce an anti-microfilarial antibody or some substance such as that postulated by Murgatroyd.[5] Some microfilariae, possibly those from ectopic sites, may be less antigenic than the majority. They may persist during treatment because they have not taken up enough of this antibody to allow it and the drug to exert fully their combined microfilaricidal potentiality. The anti-diethylcarbamazine effect of prednisone, summarized in paragraph 2, may be due to its having prevented the reaction with this antibody from occurring in a significantly greater proportion of weakly antigenic microfilariae.

The existence of a microfilaricidal antibody is implied in the work of Knott[14] and Hawking.[15] The evidence in their papers suggests that, when transfused into untreated patients who had clinical evidence of past filariasis, microfilariae disappeared from the peripheral blood much more rapidly than when transfused into nonfilarial volunteers, in whose blood they were found sometimes to persist for many weeks.

Franks[16] believed that filarial patients showed evidence of the existence of an antibody specific against microfilariae and of a separate antibody against adult worms. The other known antibody in filariasis that should be considered is the one that fixes complement.[17] This is often not demonstrable in *W. bancrofti* infections.[18, 19] The complement-fixation test is often negative also in long-infected untreated cases of loaiasis, and more often among patients with than patients without demonstrable microfilariae.[20] Thus, it may be that complement-fixing antibody is produced by every patient throughout the length of his filarial infection but is not demonstrable serologically in those cases where it has all been taken up by microfilariae. Another possibility is that Murgatroyd's substance exists but is one that has not yet been found by any serological technique.

It is evident that much more work is needed on host-parasite relationships and immunological mechanisms in filariasis. Hawking's remark in 1950 is still true: "At present it is not possible to explain this phenomenon of the persisting minority (of microfilariae) but no explanation of the action of hetrazan will be complete unless it does."

SUMMARY

Prednisone in ordinary dosage has been found to increase significantly the number of *Wuchereria bancrofti* microfilariae that persist in the night blood during treatment with diethylcarbamazine. This effect ceases when the prednisone is discontinued. The implications of this on theories concerning host-parasite relationships in filariasis and the microfilaricidal action of diethylcarbamazine are discussed.

ACKNOWLEDGMENTS

We are very grateful to Mr. W. Race, Department of Public Health, and Mr. M. Heywood, Department of Native Affairs, Cape Gloucester, for their hospitality and assistance; to Drs. C. H. Campbell and L. N. Young for access to patients in Port Moresby Hospital and to Dr. R. F. R. Scragg, Director of Public Health, for permission to publish.

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TREATMENT OF TAPEWORM INFECTION WITH DESASPIDIN, A NEW PHLOROGLUCINOL DERIVATIVE ISOLATED FROM FINNISH FERN*

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A previously unknown phloroglucinol derivative, desaspidin (Figure 1), was isolated by Aebi, Kapoor and Büchi[1] from the Finnish Broad Buckler fern (*Dryopteris austriaca* (Jacq.) Woynar). The chemical constitution has been confirmed by synthesis.[2] The acute oral LD_{50} for mice is 260 mg/kg. Desaspidin has been demonstrated to be present in Finnish but not in Swiss and Austrian *D. austriaca* roots.[3] The commercial Finnish Aspidium Oleoresin (*Extractum filicis*) also contains varying amounts of desaspidin.[4] The Finnish oleoresins of aspidium are usually employed in doses of 2.5 to 3.5 g and the corresponding Central European oleoresins in doses of 8 to 10 g, although they have the same "crude filicin" content. Hence, it is possible that the more powerful anthelmintic effect of the Finnish oleoresins is due to their desaspidin content. If this is so, desaspidin should be an active agent against cestodes.

METHODS

The series of patients at the beginning of the investigation consisted chiefly of children under 15 infected with fish tapeworm (*Diphyllobothrium latum*). Adult subjects were treated at a later stage after the method had been worked out. The courses of treatment were administered at elementary schools under primitive conditions to 50 to 150 persons on the same day. There was no previous experience of how such large-scale treatments should be organized and the method was consequently worked out and improved as the investigation progressed.

At first, magnesium sulfate was used as the cathartic. It required the addition of taste-improving substances because up to 75% of the children vomited after taking it. When its taste had been corrected, the children could tolerate 30 g of magnesium sulfate in about 200 ml of water, but the purgative effect was not satisfactory. The author, therefore, began to use castor oil emulsion as the cathartic. By this time the practical experience gained from the desaspidin courses had resulted in the following scheme of treatment:

The patients were instructed to eat a light meal at noon. After that, they were told to abstain from solid food but were allowed to have water, tea and coffee. No attempt was made to empty the bowel with purgatives or an enema before the treatment. There was no way of ensuring that the patients were co-operating during this pre-treatment period.

Before starting the treatment, fecal smears were examined microscopically for worm ova to decide whether the patients actually harbored fish tapeworm.

Between 6 p.m. and 10 p.m. the patients received a single dose of 200 or 400 mg of desaspidin in tablet form (Rosapin®, each tablet containing 50 mg of desaspidin).

Two hours after taking the tablets, 30 ml of castor oil emulsion was administered. The emulsions were prepared in 100-ml flasks immediately before use by shaking vigorously 30 ml of Aromatic Castor Oil, N.F.X. containing 0.2% Tween 60®, with roughly 30 ml of plain water.

After the castor oil had produced diarrhea an enema was given the following morning to those patients in whose stools no expelled tapeworms could be detected. After some 400 patients had been treated according to this scheme, the enemas were omitted completely as they were found to have no notable effect on the final result.

The patients' stools were examined microscopically for worm ova on the 14th day after treatment. For various reasons some of the examinations had to be performed earlier or later than this or even had to be omitted altogether.

* The investigation was performed in 1959–1960 during the Finnish Red Cross campaign against the tapeworm scourge in Northern Karelia with the permission of the State Medical Board of Finland. The desaspidin was supplied by the drug manufacturer, Medica Ltd., Helsinki, Finland.

RESULTS

Table 1 shows the doses of desaspidin administered and the age distribution of the patients.

Of the 2,368 patients, one 51-year-old man, after taking 200 mg of desaspidin and 30 g of castor oil emulsion, had diarrhea of such severity that he required hospital treatment for 8 days.

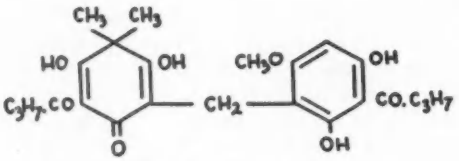

FIG. 1. Desaspidin

TABLE 1

Desaspidin dosage and the patients' age

Desaspidin (mg)	Number of persons treated				Total
	5–10 years	10–15 years	Over 16 years	Age not noted	
100			10		10
200	116	506	568	45	1235
300		1	8		9
400	160	551	318	74	1103
500			1		1
600			10		10
Total	276	1058	915	119	2368

In another 8 cases the patients complained of headache, pain, fatigue, etc. during or after the treatment.

Magnesium sulfate was the cathartic in the first 1,092 courses of treatment. It had a purgative effect in only about half of the cases. In spite of this, the stools were negative for worm ova in 75.5% of 662 children examined who had received 200 mg and in 62.3% of 210 children examined who had been given 400 mg of desaspidin. Thus, about 500 school children received desaspidin in this series without any resultant purgation. No side or toxic effects were noted.

Table 2 shows the results of 1,276 courses of treatment in which desaspidin and castor oil emulsion were used. The stools of 442 of the 573 patients given 200 mg and of 603 out of the 703 given 400 mg of desaspidin were examined for worm ova. The patients given 200 mg of desaspidin and examined 7 to 14 days after the treatment were chiefly adults; whereas, only about 50% of the patients in the corresponding group receiving 400 mg were adults. In spite of this difference between the two series of patients, the stools were found to be negative for tapeworm ova practically equally often after 200 mg and after 400 mg of desaspidin. Even when all patients whose stools were tested for ova are taken into consideration, the difference in the anthelmintic effect of the 200 mg and 400 mg doses of desaspidin was of no practical significance. The correlation between the preliminary

TABLE 2

Examinations for worm ova after the desaspidin courses of treatment

Time lapse between treatment and examination for ova (days)	Patients' age (years)	200 mg desaspidin and 30 g castor oil emulsion			400 mg desaspidin and 30 g castor oil emulsion		
		No. stool samples examined*	Stool samples free of ova		No. stool samples examined*	Stool samples free of ova	
			No.	%		No.	%
7–14	5–10	4	4		31	27	
	10–15	3	3		162	153	
	Over 16	298	269		170	157	
	Not noted	0	0		31	31	
		305	276	90.5	394	368	93.4
1–6		5	5		0	0	
Over 14		132	107	81.1	209	181	86.6
Total		442	388	87.8	603	549	91.0

* 1 stool sample was examined per patient.

TABLE 3

Preliminary versus final results of the desaspidin courses of treatment

	200 mg desaspidin and 30 g castor oil emulsion		400 mg desaspidin and 30 g castor oil emulsion	
	Cases worms expelled	Cases worms not expelled	Cases worms expelled	Cases worms not expelled
Preliminary result (= expulsion of worm during the course)	156	149	257	137
Final result at the examination for worm ova 7–14 days later				
No ova found	139	137	243	125
Ova found	17	12	14	12

results estimated by expulsion of worms during the course of treatment and the final results estimated on the basis of worm ova in the stools is shown in Table 3.

DISCUSSION

In the present series of 2,368 cases of fish tapeworm infection treated with desaspidin, objective side effects were noted in one patient and subjective ones in eight. In the first phase of the investigation, when magnesium sulfate was used as the cathartic, no side effects were observed in the approximately 500 persons who were unaffected by the purgative. The feeling of tiredness and lassitude which is practically always experienced after treatment with oleoresin of aspidium was lacking almost without exception after desaspidin treatment. Desaspidin has hardly any taste at all. The tablets were, therefore, easy to take and caused no unpleasant sensations. In toxicity and side effects, then, desaspidin is considerably better than oleoresin of aspidium, the potential systemic toxicity[5] of which is attributable to the absence of reliable chemical and pharmacological methods of standardization.[6]

Many difficulties are met in assessing the usefulness of an anthelmintic against *D. latum*.[7] The presence of a worm with scolex in the feces does not guarantee that all the parasites have been expelled since the patient may harbor several parasites. In the present investigation, 270 m (823 ft) of worm was expelled from a schoolboy and 18 scolices were demonstrated. An adult man had 16 scolices and a total of 330 m (1,006 ft) of worm. Table 3 shows, moreover, that there was no correlation between the finding of expelled worms during the desaspidin courses of treatment and the results of the examinations for worm ova in the feces 7 to 14 days after the treatment.

Microscopic examination for worm ova in the stools is of decisive importance in assessing the result of a treatment of *D. latum* infection. At what time the examination should be made is the problem. Kuhlow,[8] who summarized the literature on experimental *D. latum* infection in man, stated that worm ova were detected in the stools on the 15th day after the administration of plerocercoids. Hence, it is important to examine the stools for worm ova during the first 2 weeks after the treatment in order to rule out reinfection. This is very important in the Finnish tapeworm districts as it is never possible to exclude the possibility that the patients have been reinfected on the day of or the days after treatment. A positive finding of worm ova in the stools on the first few days after treatment is of no value since ova may have remained in the intestine although the helminths were expelled or digested. If the patient suffers from constipation it might take up to a week before all the ova of already expelled worm disappear from the intestine. Examinations for worm ova must consequently be performed preferably 7 to 14 days after the course of treatment. The results are naturally more reliable if stool specimens are taken several times during this period. One stool sample was examined per patient in the present investigation. It will be seen in Table 2 that the relative number of stool samples negative for worm ova in the courses with 200 mg and 400 mg of desaspidin was greater when the examinations were carried out on the 7th to 14th day than when they were performed later than 14 days after treatment (90.5 and 93.4% stool samples free from ova against 81.1% and 86.6%, respectively). It seems likely that reinfection occurred in the groups examined later.

It will be seen from Table 2 that treatment of fish tapeworm with 200 mg of desaspidin and 30 g of castor oil emulsion had a good anthelmintic effect. Doubling the desaspidin dose did not

improve the effect. The cathartic's important influence on the result of the treatment suggests that desaspidin did not always kill the tapeworm. The poor purgative effect of magnesium sulfate was surprising. The patients tolerated the aromatic castor oil emulsion well and found it was easier to take than magnesium sulfate. The castor oil emulsion used broke easily because of the small quantity of emulsifying agent added; and it had, therefore, to be prepared immediately before use by vigorous shaking. Better emulsions of cream-like consistency produced a slow and incomplete purgative effect, probably because the finely dispersed oil particles were absorbed in the proximal parts of the small intestine. In ambulatory patients, the castor oil emulsion used emptied the intestine in 2 to 6 hours. The effect usually ensued after 6 to 8 hours if the purgative was ingested at night.

It was difficult to prevent the patients from eating during the immediate pre-treatment hours A small group of patients consisting of 39 military servicemen who received 200 mg of desaspidin and 30 g of castor oil emulsion probably had no chance to eat "snacks." At stool examination 14 days later, worm ova were found in one of the servicemen. The results of treatment were better than the average for all the patients treated with 200 mg of desaspidin. This emphasizes the significance of fasting in desaspidin treatment.

No systematic study has been made of whether desaspidin is effective against tapeworms other than *D. latum*. According to information received from general practitioners, some 10 cases of taeniasis have been treated with 200 to 600 mg of desaspidin. Worms were expelled in all the cases. No examination was made for scolex and no follow-up examinations were performed.

The experience gained from the therapeutic experiments described suggests that desaspidin in a single dose of 200 mg is useful and practically nontoxic as an anthelmintic in fish tapeworm infection. Just as in the case of Aspidium Oleoresin, it is important that the gastrointestinal tract is as empty as possible when the desaspidin dose is administered. If castor oil emulsion is used as the cathartic in connection with the desaspidin treatment it seems unnecessary to give an enema. Desaspidin, therefore, is suitable for ambulatory practice and large-scale courses of therapy.

SUMMARY

The anthelmintic effect of the phloroglucinol derivative desaspidin in the treatment of fish tapeworm (*Diphyllobothrium latum*) infection was studied. The drug was used in doses of 200 and 400 mg. Thirty grams of castor oil emulsion was given as a purgative. The 200 mg dose of desaspidin was effective. A total of 2,368 persons received treatment. A significant side effect was observed in only one subject.

ACKNOWLEDGMENT

The author is deeply indebted to Miss Alli Vaittinen, R.N., for her valuable assistance.

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CELLULAR RESISTANCE AGAINST SCHISTOSOMULA OF *SCHISTOSOMA MANSONI* IN *MACACA MULATTA* MONKEYS FOLLOWING PROLONGED INFECTIONS*

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As a part of an investigation on Manson's schistosomiasis in primates carried out in collaboration between the institutions credited above, a study was made of tissue reactions to schistosomula as they passed through the skin, lymph nodes and lungs of previously infected, resistant *Macaca mulatta* monkeys. Studies of this type dealing with a variety of schistosomes and hosts have been published by Watarai,[1] Vogel and Minning[2] and Kagan and Meranze,[3] and more recently by Magalhaes[4] and Lin and Sadun.[5] Observations on cellular reactions in resistant monkeys are limited to the studies of Vogel and Minning[2] and of Lin and Sadun.[5]

Macaca mulatta exhibits a favorable level of natural resistance against both *Schistosoma japonicum*[2, 5] and *Schistosoma mansoni*,[6] and shows convincing evidence of acquired resistance after overcoming these infections. Acquired resistance has also been demonstrated in the rabbit.[5] In mice, the degree of acquired resistance is of a lower order.[5, 7] Whether there is a resistance in man comparable to that of the monkey is undetermined, but analogies have been suggested.[6, 8]

Unusual features of acquired resistance against schistosomes have been noted which differ from those of bacterial and viral immunity, and even from resistance to helminths which are only transitory tissue migrants,[9] namely: (1) the parasite's ability to thrive for many years in a humoral habitat with continual exposure to host antibodies,[10] (2) the long interval needed for acquiring maximum resistance,[2, 6] (3) initiation of self cure prior to maximum resistance to new exposures,[2, 6] (4) occurrence of only partial or limited resistance in some of the exposed animals,[7, 8] (5) ineffectiveness of light infections for inducing clear-cut resistance,[6] (6) lack of correlation between hitherto described antibodies and degree of host resistance (summarized by Kagan[7]) and (7) ineffectiveness of passive transfer;[11, 12] partial success has been claimed by Sadun and Lin.[13]

These features will be discussed in the light of our pathological findings and a tentative explanation will be proposed.

METHODS

As a means of inducing resistance, adult *Macaca mulatta* were infected according to several exposure schedules with the Puerto Rican strain of *S. mansoni*. The exposures were of three general types: group I, a single heavy exposure of 1000 cercariae; group II, a combination of moderate and multiple light exposures; group III, a series of frequent light exposures. Three monkeys served for lethal-exposure controls, two receiving 5,000 and the third 1,000 cercariae. The first two died after 54 and 56 days, and the latter after 64 days. Nine resistant monkeys were incorporated in this study, and a period of 3 to 5 years lapsed between the initial and challenge exposures. Observations during the course of the infection showed that excretion of eggs had either ceased or become negligible well in advance of challenge, with the exception of one monkey. Discharge of eggs was not renewed or increased in any monkey following the first challenge (5,000 cercariae), observations continuing for 83 to 245 days after exposure (see Figures 1 and 2 of Naimark et al.).[6] Thereafter, a second massive exposure of 5,000 cercariae was given, and tissues were taken at appropriate intervals. The final exposure was administered to the skin of the thorax and four extremities, with dosage divided into five aliquots, thus permitting serial skin biopsies, which were taken at intervals from 30 minutes through the 5th day after challenge. Three of the nine resistant monkeys were sacrificed 6 days after

* This work was partially supported by Grants E-1669 and E-2631, of the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

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TABLE 1

Worm recovery from resistant monkeys at varied intervals following a final challenge, and controls

Monkey no., type	No. cercariae		Days after final challenge	Worms			
	Final challenge	Previous total*		Immature	Mature	Total	% of final challenge
Control							
34	1000	—	64	0	514	514	51
35	5000	—	54	0	721	721	14†
36	5000	—	56	0	1468	1468	29
Resistant							
6	5000	7500	6	0	0	0	—
8	5000	7500	6	0	0	0	—
9	5000	11000	6	0	0	0	—
19	5000	6000	15	86	0	86	—
22	5000	11000	15	96	0	96	—
1	5000	10500	23	473	21	494	10
23	5000	7500	22	421	27	448	9
21	5000	11500	42	503	0	503	10
29	5000‡	6000	65	0	12‡	12	0.2

* Including an initial massive challenge of 5000 cercariae.

† Monkey placed in refrigerator overnight—recovery probably incomplete.

‡ ♂ Males only.

terminal challenge at which time a maximum of schistosomula would usually be present in the lung. The others were kept for longer periods for parasitological studies, so it was necessary to take lung biopsies between 6 and 8 days after challenge in order to find cercariae in the lung. Immediately following host sacrifice, the worms were recovered by separate perfusion of liver and mesenteric vessels. Complete necropsies followed, in which selection of tissues for microscopic study was guided by fresh compression preparations.[14] Of each monkey, 18 to 22 routine sections were examined microscopically, as well as serial sections of skin, superficial lymph nodes, lung, liver and colon. These were routinely stained with Hematoxylin-Eosin; in addition, Weigert's elastica, Gram and Ziehl-Neelsen stains were used whenever indicated. In the skin and lung sections, a semi-quantitative count of schistosomula was made. For further details of the study of schistosomiasis mansoni in the primate, see Naimark *et al.*[6]

RESULTS

Parasitological Observations

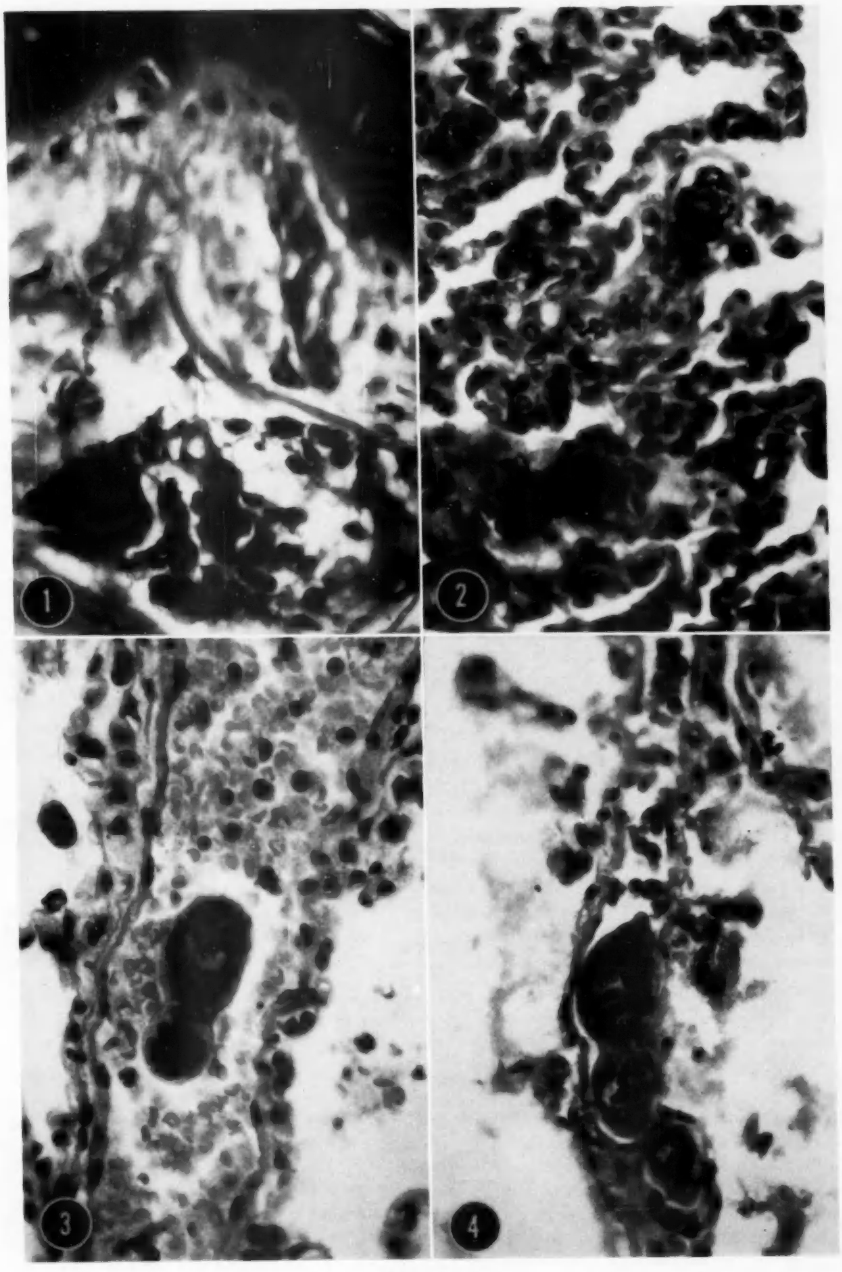
Exposures and worm recoveries, summarized in Table 1, indicate that most, if not all, worms recovered at autopsy belonged to the second, final challenge, since animals sacrificed 6 days after this exposure had no worms at all, and in monkey 29, which had a terminal exposure of only male cercariae, all the worms were male.

FIG. 1. Skin of non-resistant monkey, 72 hours after infection. Schistosomulum retained in corion. Degenerative change has occurred in the larva, a mixed inflammatory infiltrate is accumulating next to it. Hematoxylin-Eosin, × 430.

FIG. 2. Lung of non-resistant monkey, 7 days after infection. Intact schistosomulum migrating through interalveolar capillaries. There is no inflammatory reaction. The atelectasis is the result of operative procedure (Lobectomy-biopsy). Hematoxylin-Eosin, × 430.

FIG. 3. Lung of non-resistant monkey, 7 days after infection. Intact schistosomulum, free in a lung venule. Hematoxylin-Eosin, × 430.

FIG. 4. Lung of resistant monkey, 6 days after challenge. Undulating schistosomulum migrating through interalveolar capillary. Note endothelial swelling, intravascular crowding of leucocytes on both poles of larva, and mild exudation into adjacent alveoli. Hematoxylin-Eosin, × 430.



FIGS. 1–4

TABLE 2

Location of schistosomula in relation to penicilliary foci of lung in resistant and control monkeys

Type of monkey	Number monkeys	Slides examined	Schistosomula		
			Total*	Inside foci	Outside foci
Control	2	171	8	0	8 (100%)
Resistant	8	552	62	34 (55%)	28 (45%)

* Individual findings were too small to be significant.

Therefore, recovery percentages were calculated as fractions of the number of cercariae in the final exposure, and were omitted for animals killed before the 20th day following final exposure, since only part of the worms could have reached the portal habitat at that time.

Despite the limitations imposed by animal number, and by technical margin of error, three observations can be derived from the parasitological data, as a basis for subsequent histopathological interpretations: (1) The proportion of worms reaching the portal habitat is smaller in the resistant than in the normal host, suggesting that schistosomula are retained at their earlier points of migration. This effect is named the *trapping phenomenon*. (2) Many of the worms which do arrive in the portal vessels remain immature up to the 42nd day, when ordinarily all should be fully developed. This is evidence of a *stunting phenomenon*. (3) The worms reaching the portal circulation in some animals are decimated before the 65th day. This can be defined as the *premature extinction phenomenon*.

Further confirmation of the trapping phenomenon was derived from the pathological findings described below.

Pathological Observations In The Skin, Lymph Nodes And Lung

Skin. Duration and severity of cercarial dermatitis differed markedly between resistant and control monkeys. The latter showed mild dermatitis, evident mostly at 24 and at 48 hours, while the resistant monkeys had marked inflammatory infiltration as early as 6 hours, dermatitis persisting through the 5th day and longer. At its peak (24 to 48 hours) there was intra-epidermal vesicle formation, erosion and massing of polymorphonuclear leukocytes in the epidermis, followed later by hyperkeratosis, crust formation and scaling. Skin reaction was eczematoid in the resistant monkey, and fibrinoid vasculitis was seen in the corion in two instances.

Counting of cercariae in the horny and prickle cell layers and in the dermis proved unrewarding.* No clear differences were found in the speed of cercarial progression from epidermal to dermal layers, nor in the number of cercariae persisting after five days. The shifts in position observed in the sections were consistent with an initially rapid migration, followed by slower progress. Occasional larvae were retained in both resistant and control monkeys, a few in the horny layer, most in the dermis (Figure 1).

Lymph nodes. No cercariae were found. While many lymph nodes showed reticular hyperplasia,

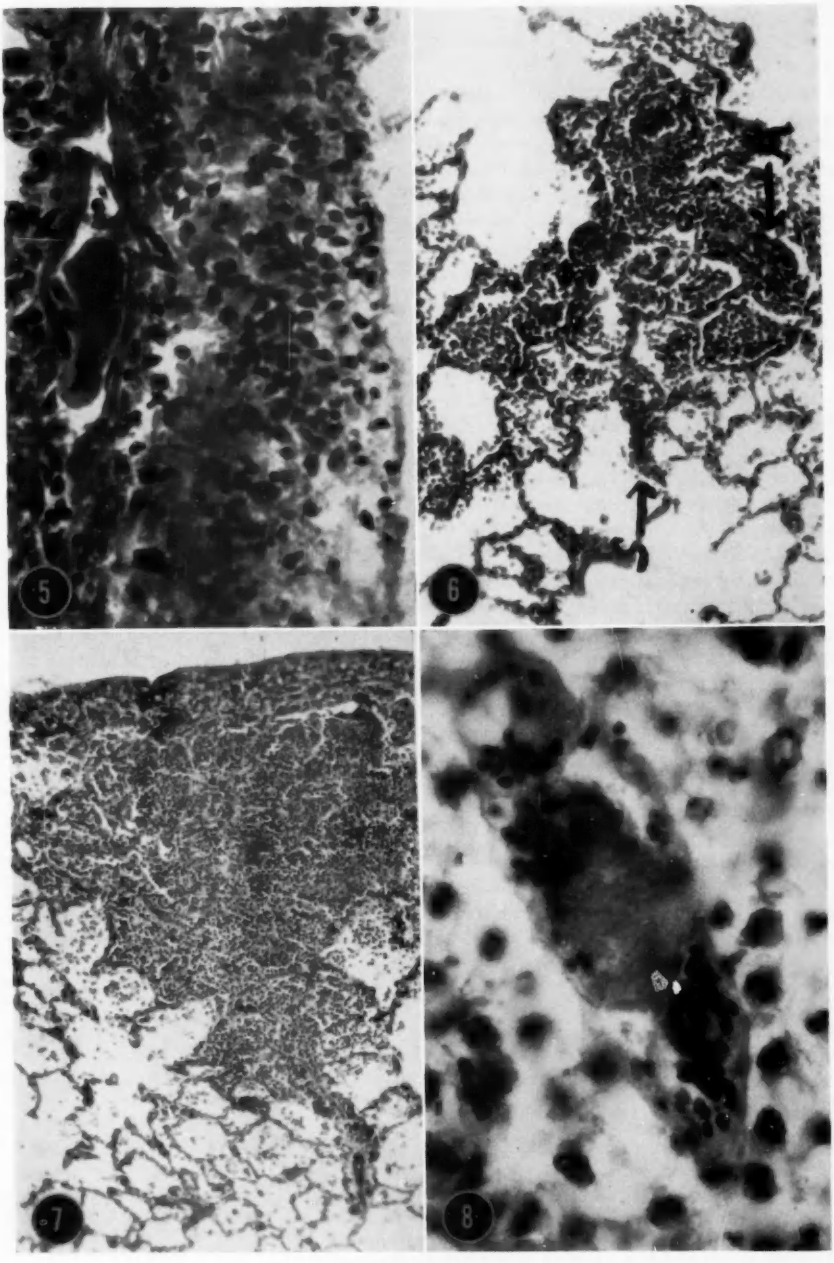
* This technique was laborious and insufficiently consistent to permit statistical analysis, especially in late stages when larvae were scarce. An oversight method based on clearing whole skin mounts was devised and proved effective in the mouse, but failed in the monkey due to greater skin thickness. Future studies should concentrate on the status at 5 days and on retention rather than velocity of penetration.

FIG. 5. Lung of resistant monkey, 6 days after challenge. Early subpleural tuft-like focus. Detailed description in text. Hematoxylin-Eosin × 430.

FIG. 6. Lung of resistant monkey, 6 days after challenge. Early tuft-like focus. S—marks the location of the schistosomulum, A—that of the feeder arteriole. Beginning consolidation is seen near that vessel, with inflammation spreading along the peripheral capillaries. Hematoxylin-Eosin, × 100.

FIG. 7. Lung of resistant monkey, 8 days after challenge. Fully developed penicilliary focus. Note pyramidal shape, extending from feeder arteriole to inflamed pleural surface and scalloped borderline. There is early fibrin deposition centrally. Schistosomulum not present in this section. Hematoxylin-Eosin, × 80.

FIG. 8. Degenerating schistosomulum trapped in lung focus. Note nuclear pyknosis, cytoplasmic vacuolization and irregularity of cuticular outline. The granular amorphous material seen in the central portion is not a constant feature. Hematoxylin-Eosin, × 900.



FIGS. 5–8

no focal necrosis was seen, nor were there fibrous scars to suggest past destruction of schistosomula.

It is to be noted that study was limited to axillary and inguinal lymph nodes at the time of autopsy, beginning on the 6th day after final challenge; whereas, lymph node passage of schistosomula usually occurs at an earlier time.

Lung. The aggregate number of schistosomula found in the control and resistant monkeys and the presence or absence of inflammatory foci around them are summarized in Table 2.

These results are based on step sections (every 5th section mounted) of autopsy- and biopsy-lung tissue, fixed by intratracheal Zenker's fluid, 6 to 8 days after final challenge.

In the *control monkeys*, schistosomula occupied the alveolar capillaries, oriented in an axial direction. Adjacent alveoli and vessels were free of mesothelial swelling of leukocytes (Figure 2). The picture suggested intimate parasite-tissue contact attended by minimal irritation. Atelectasis, when present, could be attributed to surgical procedure. Minor endothelial swelling and perivascular cuffing was seen focally, as previously described,[15] but was unrelated to the location of larvae. One section showed an intact schistosomulum in a medium-sized lung venule, oriented with the direction of flow (Figure 3).

In the *resistant monkeys*, none of the schistosomula were entirely free of inflammation. Roughly half were located within major inflammatory foci of distinctive appearance, which we named *tuft-like foci*, *i.e.*, brush-like or branching foci. The remaining half were surrounded by minor cell reactions, consisting of endothelial swelling, intravascular clumping of leukocytes, pericapillary edema, and exudation of albuminoid material into adjacent alveoli. Inflammatory changes extended both forward and backward from the larvae, within the vessel lumen. Occasionally the sinuous shape of a larva appeared molded by that of the capillary containing it, and suggested undulating motion during life (Figure 4).

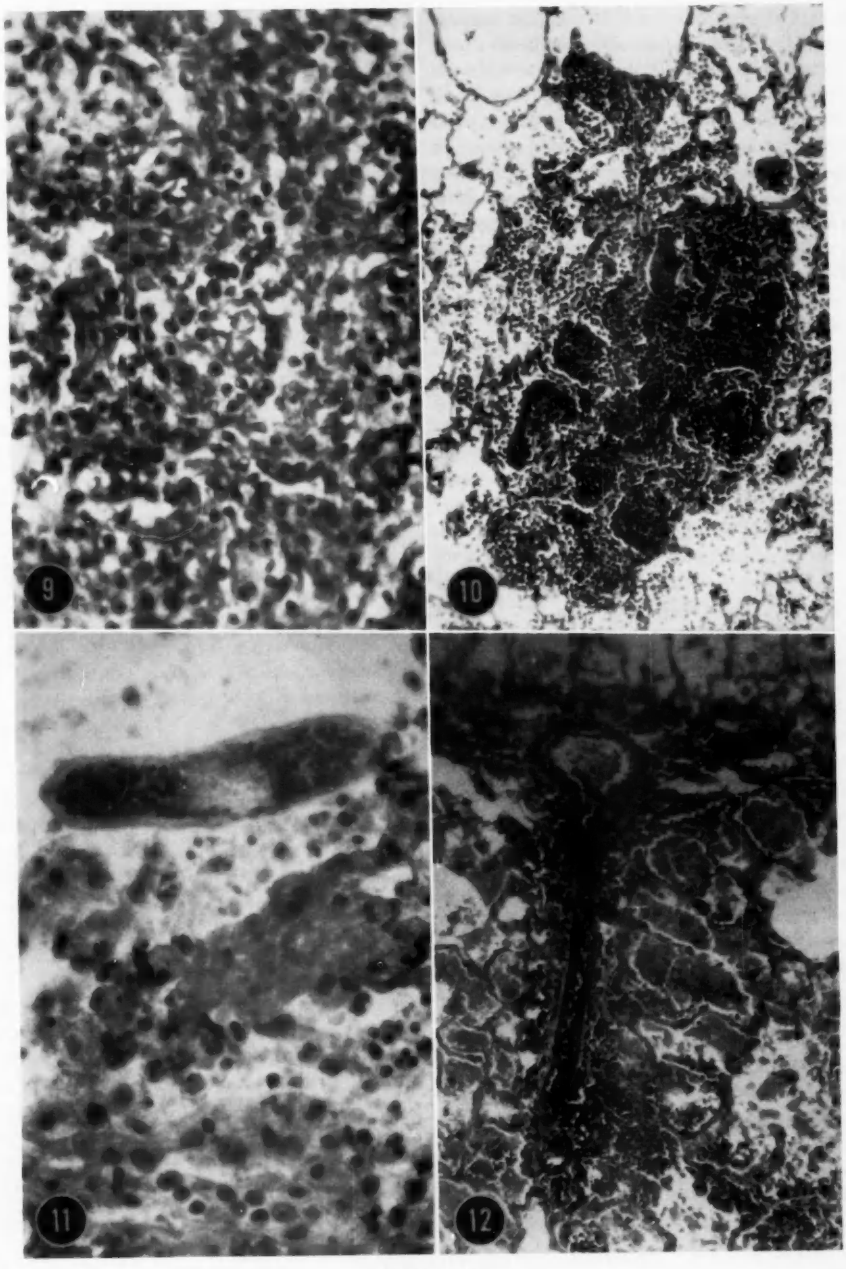
From the appearance of tuft-like foci on successive days, the following reconstruction of their course was derived: in small, early foci, schistosomula appear to be detained in precapillary arterioles or alveolar capillaries, distributed at random, but with some preference for subpleural vessels. Larvae are morphologically intact, vascular endothelia are swollen, and the lumina contain clumped leukocytes. Adjacent to the larvae, the vascular basement membranes are thickened, but not fibrinoid, the perivascular tissues are endematous and infiltrated mainly with polys, and with some eosinophils, lymphoid cells and large mononuclears. Inflammation spreads into adjacent capillaries and alveoli, the latter containing albuminoid fluid and desquamated macrophages (Figure 5). As the foci enlarge, progressive peripheral spread is evidenced by inflammatory thickening reaching out from a central feeder-arteriole into branching capillaries, while centrally located alveoli fill with neutrophilic exudate. The schistosomulum may occupy either the middle or the periphery of such foci (Figure 6), and may sometimes be seen to erupt through the capillary wall into the alveolar cavity. The largest foci, mostly on the 7th and 8th day, are club-shaped when central, or pyramidal when subpleural in location. As alveolar consolidation proceeds, fibrin is added to the exudate, with gradual increase in the proportion of mononuclears. Still, at this stage, the borders of the foci are scalloped, perivascular inflammation ad-

FIG. 9. Lung of resistant monkey, 8 days after challenge. The degenerating schistosomulum in the center of the picture can be recognized by cross-section of the ceca, and by the lumped pyknotic nuclei near its cuticle. Further alteration would make its structure unrecognizable. Note also the increasing number of mononuclears in the perilarval infiltrate. Hematoxylin-Eosin, × 360.

FIG. 10. Lung of resistant monkey, 45 days after challenge. The focus is smaller, somewhat better limited, shows dense fibrinous alveolar plugs. The schistosomulum is unidentifiable. The infiltrate has become predominantly mononuclear. Hematoxylin-Eosin, × 100.

FIG. 11. Lung of resistant monkey, 7 days after challenge. An intact larva with well-delineated cuticle and normal-appearing nuclei is seen in an alveolar space, just outside the border of a tuft-like focus. Below it there is early fibrin deposition. Hematoxylin-Eosin, × 430.

FIG. 12. Lung of resistant monkey, 7 days after challenge. The schistosomulum (not seen in this section) was located distally to the right and downward in this arteriolar branch, in an adjacent serial section, in proximity to a tuft-like focus. Note endothelial swelling and intravascular inflammatory cell aggregates throughout the length of the arteriolar branch. Hematoxylin-Eosin, × 100.



FIGS. 9–12

vancing beyond the zone of alveolar consolidation. The overlying pleura is infiltrated and thickened (Figure 7). Even with numerous serial sections, the schistosomulum cannot always be found in such foci, but when it is seen, it has lost the intravascular position observed earlier. Most schistosomula no longer appear intact, but show evidence of degeneration, such as irregular wrinkling of the cuticular outline, and clumping and pyknosis of the nuclei. A few times, precipitates are seen in the larval body, which are eosinophilic, hyaline, coarsely granular and refringent, and show no connection with the intestinal ceca. Polys contact the dying schistosomula and engage in phagocytosis (Figure 8). With increasing larval degeneration, the parasite body, embedded in dense inflammatory infiltrate, becomes difficult to identify, with only the paired ceca giving a clue to its location (Figure 9). Final disintegration was not observed, even on the 8th day. However, obviously necrotic schistosomula were seen in parallel experimental material in rabbits, confirming the impression that destruction of larvae does, in fact, occur.* Late tuft-like foci showed a mixed inflammatory cell population with many small and large mononuclears. Foci could still be identified at autopsy, 45 days after challenge. At this time, they seemed diminished in area and showed dense fibrin plugs in the alveoli (Figure 10). No foci were seen at 68 days. Scars of foci from earlier challenges were never identified in our material. Two additional features deserving demonstration were the occasional finding of schistosomula peripheral to, or just outside penicilliary foci, as if escaping (Figure 11), and the far-reaching spread of arteriolar inflammation proximal to trapped schistosomula demonstrated either in the same or in an adjacent serial section (Figure 12).

Pathological Observations In the Intestine, Liver And Other Organs

These need not be reported in detail. In essence, they confirmed the parasitological evidence of decline of infection in the resistant monkeys, as judged by the scarcity of eggs in the tissues, and by the absence of major pathological lesions in the liver, intestinal tract, lung and spleen. One monkey showed only a single residual egg-shell in the lung, none elsewhere. The remainder showed variable small numbers of non-viable eggs, sharply contrasting with the overwhelming infection of the lethal-control monkeys, which had led to hemorrhagic enterocolitis and to marked hepatic inflammation with intrahepatic portal endophlebitis. Numerous viable eggs were widely distributed throughout the organs of the controls.

* This material is to be reported separately. Lung foci in the rabbit showed less arteriolar inflammation, and an exudate composed mainly of pseudoeosinophils.

DISCUSSION

Variable mild degrees of cellular reaction against schistosomula have been observed during initial infection of several host species.[3-5] We agree with these authors that such cellular reactions are probably not the cause of natural resistance. In contrast, acquired resistance has been found to be associated with characteristically marked cellular reactions in the skin and lungs. Vogel and Minning[2] found these changes in *M. mulatta* infected with *S. japonicum*; Lin and Sadun,[5] using the same species, observed them in monkeys, rabbits and mice. Magalhaes[4] described them in the lung of mice infected with *S. mansoni*, and Kagan and Meranze[3] in the skin of mice harboring *Schistosomatium douthitti*, a natural parasite of field mice. We have described these lesions in detail as they occur in the skin and lung of *M. mulatta* made resistant against *S. mansoni*. In all these instances, while inflammation appeared both in the skin and in the lungs, proof was never given that schistosomula are retained in the cutaneous tissues of resistant animals to any greater degree than in non-resistant hosts. Our data suggest that skin retention would be, at most, a minor mechanism of acquired resistance. Lin and Sadun[5] did report, however, that the arrival of schistosomula in the lung was delayed in resistant animals.

There is general agreement that schistosomula are trapped and destroyed in the tuft-like foci found in the lungs. Vogel and Minning[2] were the first to demonstrate eosinophilic aggregates in the lung. Having recovered only a few adult worms in the portal blood, they inferred that most parasites died in these foci. Magalhaes[4] showed retention and necrosis of schistosomula in the lung but did not report on worms in the hepatic portal blood. Lin and Sadun[5] found numerous larvae trapped in the lung of mice and concluded that cellular as well as humoral responses contributed to the immune mechanism against *S. japonicum*. Our sequential study of the tissue changes en-

suing as schistosomula are trapped in the monkey and rabbit lung shows that an important proportion of challenge larvae is destroyed in this site. However, an equally considerable proportion does reach the hepatic portal system. Although the latter worms complete their migration, they fail to mature further and suffer premature extinction. The current study, in collaboration with that of Naimark *et al.*,[6] showed that many such immature worms may survive for 50 or 60 days. Once the young adults are installed in the portal vessels, no further cellular reaction ensues against them until they die and are swept into the portal radicles, giving rise to large granulomas. It must be concluded that worms in the portal vessels continue to live under the influence of host resistance, though presumably not cellular. Acquired resistance in our monkeys was achieved neither in a single site, nor against a specific stage of the parasite. The reaction was not quickly accomplished and it seemed to disturb the entire larval maturation process. Since circumlarval precipitates were not seen despite careful search, and since cellular reaction is not evident during the portal stage of worm development, the damage to the larvae may be of a metabolic order.

We propose that the distinctive features of acquired resistance against schistosomes, as listed in the introduction, can be explained best on the basis of partial blockage of host antibodies by the parasite. This is consistent with the capacity of the schistosome to survive in a humoral environment in which somatic and metabolic antibodies can be safely assumed to exist after repeated infections. The long interval needed for eliciting the effective resistance in *Macaca* and the occurrence of only partial resistance in certain animals are compatible with this concept, as are the ineffectiveness of light infections and the initiation of self-cure in *Macaca* before it can effectively suppress new infections. Both the lack of a demonstrable humoral antibody related to resistance, and the ineffectiveness of passive transfer, while they might be due to technical difficulties, are also explainable on the basis of antibody blockage. Finally, the theory provides a baseline for understanding the possible interplay of cellular and humoral factors in the trapping of schistosomula in the lungs. The concept of antibody blockage requires the demonstration of one of several mechanisms by which digenetic trematodes might defend themselves against humoral attack by the host. Such mechanisms have not yet been searched for, but several areas might profitably be explored. The permeability of epithelial or cuticular surfaces of the parasite may be sufficiently selective to exclude antibody globulin, as suggested by the extraordinary structural complexity of such membranes, revealed by electron microscopy:[16] enzymes of the worm may render innocuous such antibodies as enter its body via the digestive system. An alternative mechanism of blockage might be immunologic paralysis through antigen excess.[17]

Certain features of acquired resistance against schistosome infections are strikingly parallel to the immune mechanisms observed in millipore chamber transplants between strains of mice.[18] Intraperitoneal transplants isolated by a millipore membrane remained vital, while naked homologous tissue was destroyed. Other experiments indicated that only host cells had been excluded, not humoral elements, leading to the conclusion that tissue destruction required cellular rather than humoral agents. It need not be assumed, in the case of the schistosome, that defense is entirely cellular, and that humoral antibodies elaborated by the host are innocuous. This assumption would leave unexplained the premature extinction of parasites which have completed their migration. While it is possible that trapping of schistosomula in the lungs is purely a cellular involvement, and that the antibodies responsible for acquired resistance are cell-linked, it seems more cogent at the present time to think that both cellular and humoral factors are involved in acquired resistance, and that the observed sequence of events expresses the interplay between these two types of antibodies on one hand, and the parasite's defenses on the other.

In this context, the relative ineffectiveness of cellular reaction in the skin, as compared with the lung, might be linked to the greater mobility and vitality of the worm during the initial phase of penetration, contrasted with the more prolonged and intimate tissue-parasite association when larvae are slowed and finally trapped in the lung. Cellular attack may have weakened even those schistosomula which complete migration, leaving them stunted and exposed to humoral antibodies or antibody-like antimetabolites of

the host which they would normally be able to block.

Since immunological studies to date have failed to reveal an influence on parasite survival of any of the various antibodies which can be demonstrated serologically in schistosome infections, the antibodies related to resistance should be searched for by fluorescent staining technique, in suitable tissue specimens of resistant hosts, in order to determine whether such antibodies are, in fact, extracellular, and to which part, if any, of the worm's anatomy they attach. Further clues can be expected from studies on the anatomy and physiology of the trematode, particularly in relation to cuticular permeability and protein metabolism. Future research on immunity against schistosomes is of primary interest to both immunoparasitologists and pathologists, and requires the collaboration of both disciplines.

SUMMARY

Observations have been made on the protective mechanisms which appear to be involved in conferring a high degree of acquired resistance on *Macaca mulatta* within 2 years after initial exposure to *Schistosoma mansoni*. It has been shown that the resistant monkey develops marked cellular responses in the skin and lung during transit of schistosomula, and that many of these larvae are trapped and destroyed in the lung, where they elicit inflammatory foci of distinctive morphology which are described here in detail and named "tuft-like foci". Both the skin and the lung reactions of the resistant monkey are indicative of hypersensitivity, but only the latter appears to retain schistosomula in considerable number. In spite of lung trapping, a considerable proportion of infective larvae survives the lung stage and reaches the hepatic portal blood, but these parasites are stunted and die within 60 days after challenge without further cellular reaction except that which occurs after their death in the liver. Circumlarval precipitates were not seen around any of the multiple trapped larvae examined. The findings are discussed in the light of the literature, and it is suggested, as a working hypothesis, that (a) both humoral and cellular factors are involved in acquired resistance and (b) schistosomes may be capable of partially blocking host antibodies, possibly through absorptive control.

ACKNOWLEDGMENTS

The microphotographs for this paper were taken by Mr. Antonio Puras, University of Puerto Rico. The manuscript was read and revised by Drs. Donald B. McMullen, W.H.O., Elvio H. Sadun, and Abram S. Benenson, of Walter Reed Army Institute of Research. Dr. Gustave J. Dammin provided encouragement and valuable suggestions.

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THE ETIOLOGY OF HEPATO-SPLENIC *SCHISTOSOMIASIS MANSONI* IN MICE

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Although several factors may be involved in the pathogenesis of hepato-splenic schistosomiasis mansoni (Egyptian splenomegaly), their relative importance has not been convincingly demonstrated. It has been postulated that malnutrition,[1, 2] toxic products of worms,[3, 4] dead worms,[5-7] eggs produced by the worms,[8, 9] or a combination of two or more of these factors[10, 11] may be the cause of the disease.

Previous investigators[4-11] have been limited by the fact that each of these pathological stimuli may be the cause of lesions in the liver, therefore making it difficult to assess their relative importance. Recently, a syndrome in mice resembling human hepato-splenic schistosomiasis has been described.[12-14] The mice not only developed hepatomegaly and splenomegaly, but also showed marked elevation of their portal pressure, esophageal varices and ascites. Furthermore, the results of liver function tests[14] were remarkably similar to those seen in patients with hepato-splenic schistosomiasis mansoni. Using the development of this syndrome in mice as a criterion, an attempt has been made to separate the above etiological factors and to estimate their significance in the pathogenesis of the liver disease of schistosomiasis.

MATERIALS AND METHODS

Young adult Swiss albino female mice were exposed to cercariae of a Puerto Rican strain of *Schistosoma mansoni* according to the method of Olivier and Stirewalt.[15] Bisexual infections were established in 336 mice, at one time, by exposing each of them to 140 cercariae of both sexes. These animals were maintained in three groups: (1) on a conventional laboratory diet (Purina Chow); (2) on a fatty liver diet, deficient in protein and lipotropic substances (casein, 80 g; sucrose, 678 g; lard, 150 g; salts HMW, 40 g; B vitamins, 25 g; Ca pantothenate, 5 g; ADK solution, 15 g; L-cystine, 2 g; vitamin E in oil, 5 g); and (3) on the conventional diet with stibophen treatment at 5 weeks (250 mg/kg, bid, for 5 days).

Unisexual infections were produced in another group of mice by exposing them to either male or female cercariae, 197 being exposed to 205 male cercariae and 169 exposed to 205 female cercariae. These exposures had to be performed at different times for each of the three groups studied (5, 10 and 15 weeks) because of the difficulty in obtaining sufficient numbers of cercariae of known sex at one time. (Cercariae of known sex were obtained by infecting snails with single miracidia and testing the shed cercariae in mice.) The animals were all maintained on the conventional laboratory diet.

Following infection, various experimental procedures were performed on groups of 10 to 20 mice at 5 (6 in the case of the stibophen-treated animals), 10 and 15 weeks. A modification of the perfusion method of Yolles *et al.*[16] was used for recovery of adult worms from the portal and mesenteric veins. Egg counts were made by the method described by DeWitt and Warren,[14] and histological sections stained with hematoxylin and eosin were examined. Portal pressures were measured in mice under Nembutal® anesthesia by the introduction into the portal vein of a 22-gauge needle connected to a Sanborn pressure transducer and recording apparatus. Esophageal varices were easily seen on the serosal surface at post-mortem examination, as described by Warren and DeWitt.[12, 13]

RESULTS

Bisexual infections. The mice exposed to 140 cercariae had an average of 26 worms in the portal venous system. These consisted of an average of 10 pairs, the unpaired worms always being male (Table 1). At 5 weeks few eggs were present but by 10 weeks there was an average of 3,400 eggs per gram of liver and by 15 weeks, 5,600 eggs per gram. The liver weight, spleen size and portal pressure in this group at 5 weeks

TABLE 1

Observations on mice infected with Schistosoma mansoni*

Weeks post-infection	Type of infection	Further experimental procedures	Weight of mice (grams)	Worms per mouse	Liver weight (grams)	Total liver egg count	Spleen index length × width (mm)	Portal pressure (cm H_2O)	% of mice with esophageal varices	% deaths of mice not sacrificed for study
	Uninfected controls		24.2		1.6 ± .04†		102 ± 2.3†	5.00 ± .70†		
5	Bisexual		25.3	29 ± 1.9†	1.3 ± .05	120 ± 40†	124 ± 5.7	4.95 ± .40	0	
6	Bisexual	Treated with stibophen	25.1	0	1.7 ± .04	60	161 ± 5.9	5.25 ± .60	5	
5	Male		24.0	3 ± 0.9	1.4 ± .05	0	109 ± 5.6	5.35 ± .25	0	
5	Female		23.8	1 ± 0.2	1.4 ± .07	0	118 ± 6.9	4.95 ± .30	0	
10	Bisexual		23.6	23 ± 2.7	2.7 ± .13	9,220 ± 1,560	213 ± 8.0	10.8 ± .35	40	
	Bisexual	Deficient diet	22.2	30 ± 4.2	2.0 ± .08	5,260 ± 660	209 ± 12.1	9.7 ± .35	37	78
	Bisexual	Treated with stibophen	24.8	0	1.3 ± .07	60	110 ± 4.2	5.6 ± .50	0	
	Male		27.9	44 ± 6.3	1.7 ± .15	0	167 ± 8.6	8.0 ± .40	0	
	Female		26.9	19 ± 4.5	1.6 ± .05	0	119 ± 4.9	5.6 ± .35	0	
15	Bisexual		26.8	28 ± 6.4	2.5 ± .13	13,870 ± 3,400	286 ± 16.3	10.6 ± .86	62	53
	Bisexual	Treated with stibophen	26.7	1	1.4 ± .07	160	109 ± 4.8	5.6 ± .45	0	26
	Male		26.0	31 ± 2.8	1.7 ± .08	0	177 ± 7.8	7.0 ± .40	0	10
	Female		25.1	7 ± 2.8	1.6 ± .06	0	147 ± 10.4	6.0 ± .20	0	22

* Body weight, liver weight, spleen index and varices were means of 20 mice, whereas worm and egg counts and portal pressure were means of 10 mice.

† Standard error of the mean.

were approximately the same as in a group of uninfected controls, but at 10 weeks and 15 weeks they had increased two-fold. (Figure 1). Esophageal varices were present in 40% of the mice at 10 weeks and in 62% at 15 weeks. By 15 weeks the mortality of the mice, exclusive of those studied, was 53%.

The livers were grossly normal at 5 weeks, but by 10 weeks they were slate gray to black and were studded with numerous white granulomata (Figure 2). Microscopically, at 5 weeks there was moderate to marked periportal infiltration consisting mainly of polymorphonuclear leukocytes and eosinophils. Some pigment was noted and paired worms were seen in the larger veins. At 10 weeks many eggs were present, surrounded by typical granulomata. There was moderate to heavy periportal infiltration. No worms were seen in the liver, and a moderate amount of pigment was noted. By 15 weeks there appeared to be more granulomata but periportal infiltration had diminished. Again, no worms were seen in the liver.

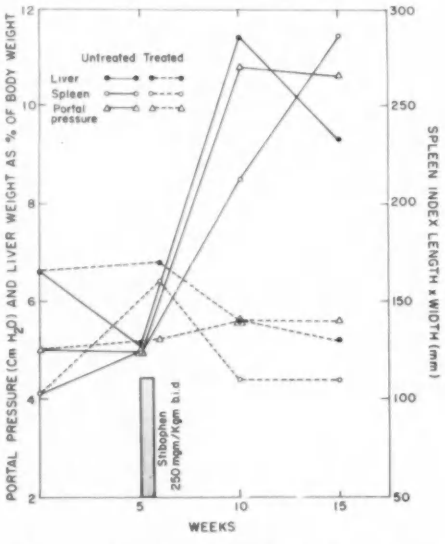

FIG. 1. The effect of treatment (stibophen) on mice with bisexual *Schistosoma mansoni* infections.

Bisexual infections on a fatty liver diet. The mice were placed on a low protein diet deficient in lipotropic substances 5 weeks following infection. Owing to the extremely high mortality they could be studied only at 10 weeks. On the basis of anatomical, physiological, histological and parasitological evidence these mice were similar to the mice infected for 10 weeks and maintained on the conventional mouse diet. The density of eggs in the liver at 10 weeks (2,760/g) was similar to that in the mice with bisexual infections maintained on the normal laboratory diet (3,400/g).

Bisexual infections treated with stibophen. These mice were similar in weight and were infected at the same time and with an equal number of cercariae as were the untreated mice. It is assumed therefore that the same average number of mature worms was present in both groups when treatment began at 5 weeks. By this time the worms had attained adult size, and had just began to oviposit.

Stibophen was injected intraperitoneally (250 mg/kg) twice daily for 5 days commencing at the 5th week. At the beginning of the 6th week there was no change in liver size, but a moderate and significant increase in spleen size was noted.

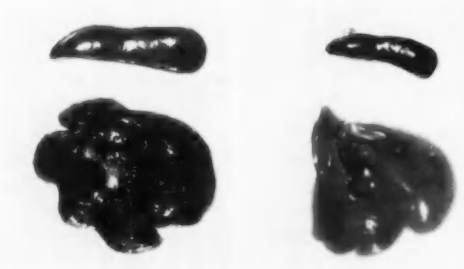

FIG. 2. Liver and spleen of an untreated mouse (left) and of a mouse treated at 5 weeks (right), 10 weeks following infection with 140 cercariae.

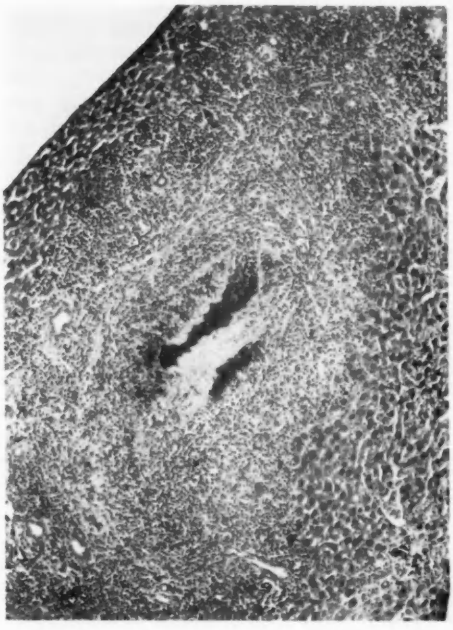

FIG. 3. Liver lesion 1 week following the onset of stibophen treatment of mice infected with 140 cercariae *6* weeks prior to autopsy. × 63.

The average portal pressure was normal, although one mouse had barely perceptible esophageal varices. At 10 and 15 weeks there was no change in liver weight, and the spleens had receded to normal size. Portal pressure remained unchanged, and no further varices were seen (Figure 1). On perfusion, occasional single worms were found. Counts revealed few eggs. The mortality, including that during the course of treatment, was approximately half that of the untreated mice.

Grossly, no liver pigmentation was noted, but occasional small hemorrhagic areas were seen (Figure 2). Sections made at 6 weeks, immediately after treatment ended, showed no parenchymal lesions nor periportal infiltration. Dead worms were seen which were necrotic and invaded by polymorphonuclear leukocytes and eosinophils (Figure 3). Giant cells were noted and there was some fibrosis. At 10 and 15 weeks there were one or two moderately large areas (1 to 2 lobules in size) of fibrosis in each section with a moderate infiltration of lymphocytes and plasma cells (Figure 4). No remains of the worms could be seen.

Unisexual male infections. Perfusions at 5 weeks revealed light infections (Table 1). There were no abnormalities in liver, spleen or portal pressure. At 10 and 15 weeks the mice were found to be heavily infected, with the number of male worms being comparable to or greater than the number of worms producing varices in the bisexually infected animals. The liver size was unchanged but there was a moderate, though significant, rise in both spleen size and portal pressure. No esophageal varices were noted. The mortality of this group was exceedingly low (10% at 15 weeks).

The livers were grossly black at 10 and 15 weeks. Microscopically, worms were seen in the larger vessels at 5 weeks, and there was marked infiltration of the periportal areas with polymorphonuclear leukocytes and eosinophils. At 10 and 15 weeks the changes were similar with an increase in pigment but at 15 weeks the infiltrate was less prominent (Figure 5). Occasional small areas of coagulative necrosis were seen.

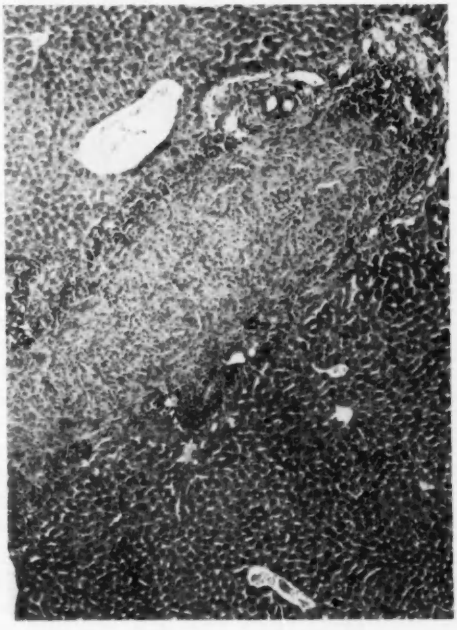

FIG. 4. Liver lesion 5 weeks following the onset of stibophen treatment of mice infected with 140 cercariae *10* weeks prior to autopsy. × 63.

FIG. 5. Liver of mouse with male *Schistosoma mansoni* infection of 15 weeks' duration. × 63.

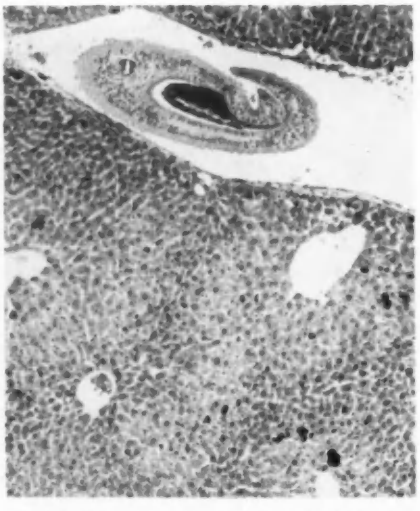

FIG. 6. Liver of mouse with male *Schistosoma mansoni* infection of 15 months' duration. × 63.

Four mice averaging 26 grams in weight were examined at approximately 15 months following exposure to 205 male cercariae. The average liver weight was 1.6 grams, 6.1% of the body weight, and the spleen index averaged 113 sq mm. The portal pressure averaged 6.3 cm of water and there were no esophageal varices. Each of the above parameters was within normal limits (cf. Table 1).

The livers were black. Microscopically, male worms were seen in the larger blood vessels and there were large amounts of pigment in the Kupffer cells. However inflammatory infiltration was minimal and there was no parenchymal necrosis (Figure 6).

Unisexual female infections. These infections were light with the exception of those studied at 10 weeks. There were no significant changes in any of the parameters measured, and the mortality was low.

The livers were lightly pigmented. Histologically, female worms were seen in some sections, and there was mild to moderate periportal infiltration consisting of polymorphonuclear leukocytes and eosinophils.

DISCUSSION

In this study an attempt was made to isolate each factor postulated as a primary cause of the development of hepato-splenic schistosomiasis mansoni. The effect of these factors on the development of this syndrome in mice was then studied.

Malnutrition. Previous investigations describing hepato-splenic schistosomiasis in mice[14] were performed while the animals were on an optimal well-balanced diet. Therefore, nutritional inadequacy did not appear to be a necessary factor in the development of this syndrome in mice. Furthermore, DeWitt[17] has shown that, in a sense, malnutrition in the early stages of infection may protect animals from the full brunt of the disease by inhibiting the development of the worms and their production of eggs. Nevertheless, in order to put the dietary question to a further test, mice were placed on a diet deficient in protein and lipotropic substances 5 weeks following exposure to cercariae (when the worms were mature and had begun oviposition) in order to determine whether malnutrition exacerbated the disease. The deficient diet had no apparent effect on the parameters of the syndrome which we were measuring.

Toxins. Several authors have claimed that toxins produced by the worms, particularly male worms,[3, 4] are a major factor in the development of hepato-splenic schistosomiasis mansoni. Microscopic examination of the livers of infected

animals has revealed not only periportal inflammatory infiltrates, but small areas of coagulative necrosis of the parenchyma.[10] Toxins are presumed to have caused this damage. However, it is not known whether these relatively mild microscopic lesions were related to the development of significant disease. Therefore, the relation of unisexual male and female infections to hepato-splenic schistosomiasis was assessed in this study.

In these experiments, no signs of hepato-splenic schistosomiasis mansoni were seen in the relatively mild female infections, but mice infected with male worms did show some changes. There was a moderate elevation of portal pressure and an increase in spleen size. However, these changes were never as severe as those seen in the presence of typical hepato-splenic schistosomiasis mansoni, and the mice returned to normal in long-term infections.

Dead worms. The contention that dead worms are a major factor in the pathogenesis of Egyptian splenomegaly[5-7] is based on results showing large areas of acute necrosis around recently killed worms, and on the supposition that this leads to the development of residual fibrosis. Magalhães Filho and Coelho[5] infected mice with large numbers of cercariae. Following treatment with stibophen, they saw acute necrosis and some residual fibrosis and therefore considered this an important factor in the pathogenesis of the liver disease of schistosomiasis. Gillman[6] considered dead worms as a more important factor than eggs in his study of two monkeys in which he noted a severe reaction around occasional dead worms. Hamilton *et al.*[7] described dead worms in liver sections obtained by wedge biopsy from patients undergoing splenectomy. These were found in only 7 of 73 cases. Most if not all of these patients had probably been treated with schistosomicidal agents prior to surgery. On the basis of this evidence Hamilton *et al.*, too, felt that dead worms may be a major factor in the production of liver disease.

In this study all worm pairs were eliminated in the treated mice, and no eggs were produced after treatment was instituted. Consequently, there were very few, if any, eggs in the livers of these animals. Control mice developed severe hepato-splenic schistosomiasis mansoni whereas treated mice had only a mild transient splenomegaly. In addition the mortality of these rigorously treated mice was about one half that of the control mice. Microscopically, parenchymal necrosis surrounding dead worms was seen in the liver soon after treatment was terminated, followed by localized fibrosis 4 weeks later. This appeared to be undergoing some regression by 9 weeks. Therefore, under the conditions of these experiments dead worms alone appear to play no significant role in the development of hepato-splenic schistosomiasis in mice.

Eggs. Bisexually infected mice in which there is egg production develop hepato-splenic schistosomiasis mansoni, whereas this syndrome is neither produced nor exacerbated (under the conditions of these studies) by placing the animals on a deficient diet, treating them with a schistosomicidal agent or infecting them with only one sex of the trematode. It is felt, therefore, that eggs are the prime factor in the development of hepato-splenic schistosomiasis in the mouse, that toxins produced by the worms or an allergic reaction to their presence may be a contributing factor in the early stages, and that dead worms and malnutrition are probably only of ancillary importance.

SUMMARY

Several factors may be involved in the pathogenesis of hepato-splenic schistosomiasis mansoni, but their relative importance has not been convincingly demonstrated. It has been postulated that malnutrition, toxic products of worms, dead worms, eggs produced by the worms or any combination of these factors may be the cause of this disease. Studies of this problem in the past have been hampered by the lack of a suitable experimental animal. Recently, the occurrence in mice of a syndrome resembling hepato-splenic schistosomiasis in humans has been reported. With the development of this syndrome in mice used as a criterion of significant disease, the effect of each of the above factors was studied. On the basis of the results obtained it is felt that eggs are the prime factor in the development of hepato-splenic schistosomiasis in the mouse, that toxins produced by the worms or an allergic reaction to their presence may be a contributing factor in the early stages, and that dead worms and malnutrition are probably only of ancillary importance.

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CERVICO-FACIAL ACTINOMYCOSIS: FIRST REPORT FROM PANAMA

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Systemic mycosis caused by the aerobic actinomycete, *Nocardia asteroides* (Eppinger) Blanchard, 1896, has been reported in only three patients on the Isthmus of Panama.[1-3] The infection was localized in the lungs and pleura of two patients, and produced a mycetoma of the left foot in the other. Systemic disease presumably due to an unidentified actinomycete was diagnosed by pathologic examination in a wild animal. *Hydrochoerus isthmius* Goldman, 1912 (Isthmian capybara),‡ that had been in captivity for 12 years.[4]

We have no knowledge of systemic mycosis caused by the anaerobic actinomycete, *Actinomyces bovis* Harz, 1877, having been previously observed on the Isthmus of Panama. The object of this report is to present the first confirmed human case of actinomycosis of the cervico-facial type, caused by *A. bovis*.

P. R., a 37-year-old farm laborer, mestizo and native of Anton Valley, was hospitalized in Santo Tomas Hospital in Panama City in August, 1960. The clinical history revealed that the patient had been suffering for 1 month with oral lesions and inflammatory nodules localized on the right malar region of the face. During the course of these 4 weeks, he experienced morning chills and general malaise. He was anorexic, asthenic, and had lost 12 pounds. The external lesion began as a small nodule on the right side of the face, and successively, above and below this site five additional nodules developed. The nodules softened and some opened spontaneously, by multiple fistula tracts, draining a foul-smelling, yellow-sanguinous fluid.

The physical examination at the time of hospitalization revealed loss of weight, pallor of skin and conjunctiva, numerous infected dental roots, and edema on the right side of the face. Edema was evident from the lower eyelid upwards, and in the right cervical region extending 10 to 12 cm above the inferior border of the mandible. There were six well-defined nodules of which four were localized in the submandibular region and two in the facial area. One of the facial and three of the cervical nodules had previously opened spontaneously. The remaining two had not opened, were not painful on palpation, and were adherent to the skin. Pus was aspirated from these two nodules for mycological study.

Macroscopic examination of the pus revealed abundant granules, characteristic of "Ray Fungus." Microscopically these granules could be seen to consist of central and peripheral portions. The central area was composed of branching filaments, and was totally devoid of spores, whereas the border of the granule was comprised of clubbed filaments. A Ziehl-Neilsen and a gram stain of the preparations, made after compressing the granules between two cover slips, revealed non-acid-fast, gram-positive filaments.

The aspirated pus was inoculated into three tubes of Brewer's thioglycollate broth. The tubes were incubated at 37°C, and after 6 days small colonies began to appear near the bottom of the tubes. The colonies increased in size and rapidly grew throughout the medium, with the exception of the top 15 mm.

Petri dishes containing brain-heart-infusion-glucose agar were inoculated with the organism recovered in the broth. The plates were incubated aerobically and anaerobically at 37°C in the presence of 5 to 10% CO_2. In 10 days white colonies 2 to 3 mm in diameter were observed in the plates incubated anaerobically. No growth was obtained by aerobic culture. Stained preparations of the organism from both broth and agar showed gram-positive branching filaments and diphtheroids. The cultural, microscopic and tinctorial characteristics of the organism were compatible with those of *A. bovis*.

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‡ The Isthmian capybara, the world's largest rodent, is native to Panama, and restricted to the Province of Darién which borders on the Republic of Colombia.

DISCUSSION

A clinical diagnosis of cervico-facial actinomycosis was made in a patient presenting numerous nodules and fistulae located high on the inferior mandibular angle, and extending below the tragus. Laboratory confirmation was obtained by the demonstration of granules of characteristic microscopic morphology and recovery of *A. bovis* from material aspirated from the nodules.

The patient received 600,000 units of penicillin and 1 gram of oxytetracycline daily for 3 weeks. The nodules and oral lesions disappeared, and the fistulae cicatrized within the period of 2 weeks.

The confirmation of the first case of cervico-facial actinomycosis, caused by *A. bovis*, demonstrates the existence of this disease on the Isthmus of Panama.

SUMMARY

The first known case of cervico-facial actinomycosis on the Isthmus of Panama was confirmed by recovery of the organism *Actinomyces bovis*.

The fungus was found to be sensitive to penicillin and oxytetracycline and the patient made an uneventful recovery.

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THE PROMOTING EFFECT OF TRICHINOSIS ON ENCEPHALOMYOCARDITIS (EMC) VIRUS INFECTION IN RATS

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The present report shows that adult rats, inoculated with *Trichinella spiralis* in doses below those resulting in apparent illness, become more susceptible to disease induced by encephalomyocarditis (EMC) virus than do normal rats, as judged by (a) a greater incidence of crippling and death, (b) multiplication of the virus in the nematode-infested muscle, and (c) a sharp and sustained loss of body weight. Adult laboratory rats are relatively resistant to infection with EMC virus under usual circumstances. Thus, a number of investigators[1-5] have reported only inapparent infections as occurring in these animals, although Kilham, Mason and Davies[6] have shown that a proportion of adult rats are, in fact, susceptible to overt disease. Suckling rats, on the other hand, are uniformly susceptible. Thus Kilham and associates showed that, whereas EMC virus multiplied in the skeletal muscle and brain of sucklings inoculated when 5 to 11 days old, little or no virus could be recovered from weanling rats inoculated in a similar manner. The implications of present findings, as they relate to the possible ecology of EMC virus infections in nature and to synergism between unrelated organisms, are considered in the discussion.

MATERIALS AND METHODS

Details of the performance of virus titrations in weanling mice and of the harvesting, preparation and storage of infected tissues are given in a previous report.[6] Additional details relating to the present experiments are outlined below.

Experimental animals. The animals used were randomly bred Sprague-Dawley and Osborne-Mendel rats and Swiss albino mice.

Virus. The strain of EMC virus employed was one originally isolated from a baboon at the Virus Research Institute at Entebbe, Uganda, and carried through six passages in mice, four in mongooses,[7] and approximately six more in mice. Seed virus used in present experiments consisted of a pool of infected mouse brains. This pool had an LD_{50} of 10^{-7} when titrated in the brains of weanling mice, the calculations being made by the method of Reed and Muench.[8] Rats received seed virus in 1-ml volumes given intraperitoneally in dilutions of either 10^{-2} or 10^{-4}. These dilutions are referred to as being the maximal and minimal doses of EMC virus, respectively.

T. spiralis. Larvae for infection were taken from animals which had been infected not less than 6 weeks previously. All but two of the experiments were done with a strain of *T. spiralis* derived from pork obtained through the courtesy of Mr. C. W. Hill of the Department of Agriculture, and maintained in rats. The other two experiments were done with a strain which was maintained in the laboratory in mice. Infested meat was minced and digested at 37°C for 3 hours in 15 volumes of artificial gastric juice which was stirred constantly during the procedure. Following digestion, the mixture was strained through gauze, sedimented, and washed with 0.85% NaCl. The larval suspension was then diluted with saline and three 1-ml samples were counted to measure the larval density. A volume of the suspension calculated to contain the desired number of larvae for each rat, based on its weight, was then delivered into a conical centrifuge tube. After sedimentation, the larvae were taken up into 0.2 or 0.3 ml of saline and injected into the rat's esophagus by means of a tube made from a bent and blunted 2-inch 18-gauge hypodermic needle.

RESULTS

Organization of experiments. The experiments presented below were organized on a basic plan in which rats of approximately 2 months of age and of the same sex were divided into three groups

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of equal size. Animals in groups 1 and 2 received *T. spiralis* larvae in a dose of 18 to 20 larvae per gram of body weight. After an interval of 10 days, the rats of group 2 were given EMC virus by intraperitoneal inoculation, this interval coinciding expectedly with a migration of large numbers of larvae into a skeletal muscle. A third group of rats was inoculated with an equal amount of virus at the same time. In this manner the final disposition was that group 1 had received *T. spiralis* alone; group 3, EMC virus alone; and group 2 had received both agents. All rats were weighed every 3 or 4 days. Although the experiments were terminated 3 weeks after inoculation with virus, a number of group 1 and 2 animals were checked after an interval of 3 or 4 months in order to determine the degree of their infection with encysted larvae. Counts made at this time indicated that there were from 500 to 4,700 larvae per gram of rat skeletal muscle.

The three groups of rats, *i.e.*, those infected with *Trichinella*, those with viral infection, and those with both, were compared on the basis of prevalence of crippling and death, body weight, titrations of virus from tissues, and histological examination of skeletal muscles.

TABLE 1

Incidence of disease and of death among male and female rats in all experiments performed

Sex	Condition of rats	*T. spiralis* group 1	*T. spiralis* + EMC virus group 2		EMC virus group 3	
		T. spiralis alone*	Max dose†	Min dose†	Max dose of EMC	Min dose of EMC
Male	Inoc.	40	19	22	20	22
	Crippled	None	18	14	9	3
	Died	None	9	5	2	2
Female	Inoc.	90	24	22	24	22
	Crippled	None	21	14	8	None
	Died	None	8	4	1	None

* Dose = 20 larvae per gram of rat, given by stomach tube.

† Dilutions of encephalomyocarditis (EMC) virus were given to rats i.p. in 1-ml volumes. The maximal dose equaled 33×10^8 and minimal dose 33×10^2 LD_{50}'s of virus in terms of titrations made by i.c. inoculation into weanling mice.

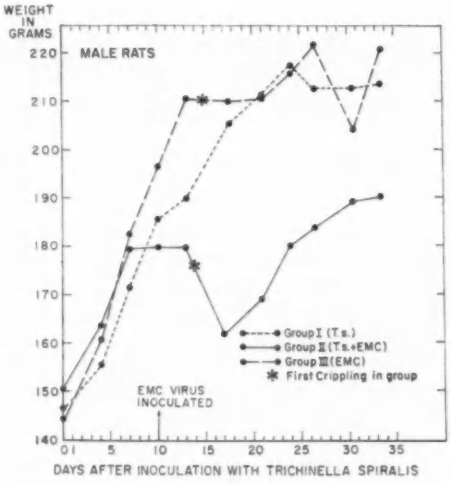

FIG. 1. Successive average weight determinations of rats of a single experiment, inoculated with *Trichinella spiralis*, encephalomyocarditis (EMC) virus, or a combination of both. (Doses of *T. spiralis* = 20 larvae/g and EMC, 33×10^5 LD_{50} for weanling mice i.c. Only animals which survived are included in the calculations.)

Prevalence of crippling and death. Crippling is used in the following discourse as a general term to describe abnormal locomotion ranging from a dragging of one foot to complete helplessness. Since only gross observations were made, one could not determine whether crippling was due to muscle damage, nerve damage, or to combinations of both.

Table 1 shows clearly that while infection with *T. spiralis* caused no crippling or death among group 1 rats and EMC virus alone caused relatively little crippling or death, especially when given in a minimal dose, the presence of both agents together resulted in a comparatively high incidence of both death and disease.

There was a sex difference in response to uncomplicated EMC virus infection in that male rats had a higher prevalence of crippling and deaths than did the females. This difference was a constant finding among group 3 rats. No such difference was obtained among group 2 rats in which disease was induced by the combined infections with nematode and virus.

Comparisons of body weights. Illustrative data on determinations of body weights are sum-

marized in Figure 1. Group 1 and group 3 rats usually grew at about the same rates throughout the course of the various experiments, although those of the former group, which had *T. spiralis*, sometimes fell slightly behind. On the other hand, the group 2 rats with the combined infection began to lose weight about 3 to 5 days after receiving the virus. The animals did not regain their pre-infective weight until a week or so later and at the end of the experiment their weight was still below that of the other groups.

The cause of the weight loss experienced by the group 2 rats cannot be stated with certainty. It was apparent, however, that the entire mass of skeletal muscle was greatly diminished. This could have resulted from direct damage to the muscle or, at least in part, to a failure of the diseased rats to take food and water. No record of food or of water intake was made.

Titration of virus in tissues. Table 2 gives comparative determinations of the extent of viral multiplication in tissues taken from rats with and without trichinosis. A striking feature was the finding that EMC virus occurred in relatively high titers in skeletal muscles which contained migrating and developing Trichinella larvae while no virus was found in corresponding muscles taken from rats injected with the virus alone.

Histology of infected skeletal muscles. Histological examinations were made on the skeletal muscles of 25 rats from the three experimental groups. The sections of muscle studied were taken from the back of the neck and from the thigh 3 to 7 days after the inoculation with virus which in turn was 13 to 20 days after the inoculation with *T. spiralis*. The virus was at its peak titer at this time. While no quantitative studies were attempted, the following histology was observed. First, rats inoculated with EMC virus alone had essentially normal muscles; second, animals given *T. spiralis* alone had lesions characteristic of early trichinosis with developing larvae and degenerating muscle fibers interspersed among fibers of normal appearance; and, finally, the muscle sections of the rats harboring both agents were similar to those of the rats with

TABLE 2

Titrations of encephalomyocarditis (EMC) virus in tissues from adult female rats with and without trichinosis

Agents inoculated	Day tissue harvested after virus inoc.	Experiment A					Experiment B				Condition of rats
		Brain	Blood	Heart	Neck mus.	Leg mus.	Brain	Blood	Heart	Leg mus.	
*T. spiralis** + EMC†	1						<1	<1	None	None	Well
	3	4.7‡	1.5	1.0	5.0	3.0	3.5	None	<1	3.7	Exp. A, crippled; exp. B, well
	5	3.2	1.5	1.2	5.0	4.7	2.2	None	None	5.2	
	7	2.5	None	None	2.4	1.3	2.3	<1	<1	4.8	Crippled
	10	None§	None	None	2.8	None					Crippled
	14						None	None	None	<1	Crippled
EMC only	1						0	None	<1	None	Well
	3	None	None	None	None	None	<2	None	None	None	Well
	5	4.0	None	None	None	None	4.0	None	None	None	Crippled
	7	2.0	None	None	None	None	3.5	<1	None	None	Crippled
	10	1.7	None	None	None	None					Crippled
	14						<1	<1	None	<1	Crippled

* Given orally 10 days before EMC virus, in dosage of 20 larvae per gram of rat.

† EMC virus given in maximum dose of 33×10^5 LD_{50}'s in terms of titrations made by i.c. inoculation into weanling mice.

‡ Titers expressed as inverse log of dilution killing 50% of mice inoculated.

§ None = no virus isolated from a 1:10 suspension.

TABLE 3

Results in terms of crippling and disease in adult female rats given encephalomyocarditis (EMC) virus at varying time intervals after inoculation with Trichinella spiralis

Group*	Time EMC virus† given after *T. spiralis*‡								
	9 days			27 days			61 days		
	1	2	3	1	2	3	1	2	3
Inoc.	30	10	10	19	10	9	9	10	7
Crippled	None	8	None	None	4	None	None	None	None
Died	None	3	None	None	3	None	None	None	None

* Rats in these groups were inoculated with the following agents. 1 = *T. spiralis*, 2 = *T. spiralis* + EMC, 3 = EMC.

† EMC virus given in minimal i.p. dose = $33 \times 10^3 LD_{50}$ for weanling mice i.c.

‡ Given in dose of 20 larvae per gram of body weight.

T. spiralis alone except for the evidences of a greater cellular response. There was no histological evidence of damage to heart muscle in rats from any of the three groups.

Relation of age of Trichinella *infection to disease induced.* In all of the experiments described above, EMC virus was inoculated when the *Trichinella* infection was 10 days old. Two additional experiments were performed to determine whether similar results in regard to crippling and death could be obtained when the time interval between the exposure to *T. spiralis* and to EMC virus was increased. In one experiment recorded in Table 3, EMC virus was given at 9, 27 and 61 days after administration of *T. spiralis*. The data indicate that administration of virus as late as the 27th day still produced gross evidence of injury whereas control animals given the virus alone remained unaffected. Viral infection at 61 days had no effect. In a second experiment, in which the maximal dose of virus was used 4 weeks after inoculation with *T. spiralis*, five of five rats which had received the dual infection became crippled and three of them died.

Incubation periods. When crippling occurred in rats inoculated with virus alone, the onset of the disease was generally a few days later than the onset of crippling among the group 2 rats. Exact data were difficult to obtain, however, since crippling might be doubtful for a day before becoming obvious. A demonstration of the later onset of disease in the group 3 rats, however, was provided by an experiment in which the minimal dose of virus was used (Figure 2). It will be seen in this figure that the onset of crippling and deaths occurred substantially earlier among rats with the combined infection.

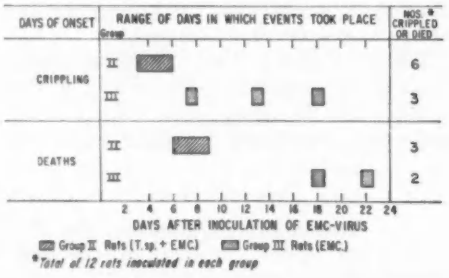

FIG. 2. Comparison of days of onset of crippling and of deaths in rats, with and without trichinosis, which had received the minimal dose (see text) of encephalomyocarditis (EMC) virus.

Variation of effect with dosage of Trichinella. The number of *Trichinella* larvae given in the usual experiment was chosen arbitrarily but was large enough to assure that there would be numerous larvae migrating to the muscles when the virus challenge was given. When it was learned that this concentration of migrating larvae did increase the injury to the rats after virus challenge, it appeared desirable to learn whether a smaller number of worms would produce similar results. In one experiment, therefore, the number of larvae fed to the rats was varied (Table 4) so that 5, 10 or 20 larvae per gram were given to different groups of otherwise comparable rats. It can be seen from the table that the two larger dosages of larvae had an appreciable effect on the prevalence of overt disease after virus chal-

TABLE 4

Effects of varying doses of Trichinella spiralis *on the crippling and deaths associated with a concurrent infection with encephalomyocarditis (EMC) virus*

Group	1	2			3
Agents inoc.	*T. spiralis*	*T. spiralis* and EMC virus*			EMC virus*
Dose of *T. spiralis*	20 L/g†	20 L/g	10 L/g	5 L/g	—
Inoc.	8	9	9	9	10
Crippled	None	8	7	3	5
Died	None	4	2	2	1

* Given in the maximal dose of 33 × 10^6 LD_{50}'s for weanling mice inoculated i.c. This dose was given 10 days after the inoculation with *T. spiralis*.

† The doses of *T. spiralis* are recorded in numbers of larvae per gram of body weight of rats inoculated.

lenge while the smallest dosage of larvae had none.

DISCUSSION

The present study shows that *T. spiralis* promotes a proliferation of EMC virus in rat skeletal muscle, although it does not explain the underlying mechanism. It is conceivable that physiopathological changes accompanying invasion and encystment of *Trichinella* larvae produce localized conditions favorable to the virus. An experiment of interest in this connection is one in which Rowe[9] promoted a multiplication of group A Coxsackie virus in adult mouse muscle by sectioning the corresponding nerve. Rowe cited evidence that denervation may make adult more like infant muscle in regard to certain enzymatic activities.

The concept that one parasite can alter the response of a host to a second and unrelated one has been demonstrated by a number of investigators. Thus, Niven *et al.*[10] and Gledhill and Dick[11] showed that mouse hepatitis virus (MHV) and the protozoan *Eperythrozoon coccoides* can induce hepatitis in mice when given as concurrent infections, but not when either is given separately. An additional example of synergism in disease has been provided by Mochizuki, Tomimura and Oka,[12] who provoked encephalitis by giving mice eggs of the nematode *Toxocara canis* by mouth and then the virus of Japanese B encephalitis by subcutaneous inoculation. Neither of these agents was pathogenic when given alone by these routes.

The ecology of EMC virus infections is relatively little known. Studies by Kilham, Mason and Davies[7, 13] in an endemic area in Africa indicated that the virus might spread among wild mammals such as field rats and mongooses by ingestion of infected carcasses. The role of rats of the genus *Rattus* in the cycle of transmission, however, has not been clear. It is curious that these fairly resistant species, which include both the black (*R. rattus*) and the Norway (*R. norvegicus*) rats, should be the ones in which circulating antibodies against EMC virus have been most often encountered.[4, 14, 15] One possibility is that resistant species may serve a special role in maintaining a reservoir of infection. This situation has been well demonstrated, for example, by Baltazard and Bahmanyar[16] in relation to plague. On the other hand, present studies indicate that under conditions such as concurrent parasitic infection a resistant species may be converted into a susceptible one which would be more likely to transmit infection to other animals.

SUMMARY

Young adult rats given a combined infection with *Trichinella spiralis* and encephalomyocarditis (EMC) virus experienced a high incidence of crippling and death while control rats which received the nematode alone remained free of disease. Rats given the virus alone had a much lower incidence of crippling than did those with the combined infection.

Rats which received *T. spiralis* and EMC virus were also distinguishable by a sharp and sustained loss of weight, presence of virus in skeletal muscle and by some differences in muscle histology.

The synergistic effect of *T. spiralis* on EMC virus infection was demonstrable over a period of 10 to 28 days after inoculation with the nematode.

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AN ETIOLOGIC STUDY OF SEASONAL ASEPTIC MENINGITIS AND ENCEPHALITIS IN THE CENTRAL VALLEY OF CALIFORNIA*

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The Central Valley of California has long been recognized[1-6] as an endemic area for two of the arthropod-borne viral encephalitides—Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE). From 1945 through 1958, 667 laboratory-proved cases of WEE and 483 cases of SLE were listed in the records of the California State Department of Public Health. In most years, however, these two entities accounted for less than one-third, often for less than one-tenth, of the cases of acute infectious encephalitis reported in the State. Thus, of 2,517 cases of infectious encephalitis reported from 1953 to 1957, approximately 39% were attributed to mumps virus infection, 2% to WEE virus, 6% to SLE virus, and 18% to various other infections such as measles, rubella, chickenpox, influenza or pertussis; but 34%, representing an average of some 170 cases per year, were recorded as "etiology undetermined."[3, 4] Cases of "undetermined etiology," including many patients serologically negative for both WEE and SLE, occurred predominantly during the summer months and tended to parallel the seasonal distribution of arthropod-borne encephalitis. The recognition in recent years that enteroviruses of the Coxsackie and ECHO groups are frequently the incitants of aseptic meningitis[7-9] and occasionally of more severe encephalomyelitic illness,[9-12] suggested that these viruses might account for some of the encephalitis of undetermined etiology in California. Accordingly, during the summer months of 1956, 1957 and 1958, laboratory studies to detect enterovirus infections, in addition to the usual encephalopathogenic viruses, were undertaken in cases of suspected viral disease of the central nervous system (CNS) observed in selected hospitals in the Central Valley. Study was directed broadly at the entire spectrum of acute febrile CNS disturbances in order to compare the association of enteroviruses and the arthropod-borne encephalitis viruses with various clinical types of CNS disease.

The results of the investigations made during these three consecutive summers are summarized in the present communication.

* The work on which this paper is based was supported by a grant (E-1475) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

MATERIALS AND METHODS

Selection of patients. The investigation was planned to provide uniform data on all clinical types of acute infectious CNS disease. The clinical criteria for selection of patients for study were the presence of fever and any signs of CNS involvement. Clinical data and specimens for laboratory study were obtained in conjunction with the California Encephalitis Surveillance Program.[6] Through the co-operation of local health officers and hospital personnel, similar observations were made in each of four health jurisdictions in the Central Valley—Kern County, Fresno County, San Joaquin County and Sutter-Yuba (bi-county) Health Departments. A medical student, working under the direction of a medical officer of the State Health Department, was assigned to each jurisdiction to assist in obtaining specimens and recording clinical findings. From approximately mid-June through the first week of September, an effort was made to screen all patients admitted to the infectious disease unit of the county hospitals in these jurisdictions and to maintain current rosters of those patients having fever and signs of CNS involvement. Cases of acute infectious CNS disease were only occasionally admitted to private hospitals in these four jurisdictions, but such cases were also included in the study group whenever possible. In each case an attempt was made to collect 2 or 3 stool specimens at 24- to 48-hour intervals, and 3 serial blood specimens—on admission, and 14 days and 4 to 6 weeks post-onset.

A case history form summarizing the clinical observations was completed for each case.

Official morbidity reporting. Although this study of hospitalized cases very likely stimulated local interest and aided in case-finding of suspected CNS diseases, the regular administrative procedures by which official reports of infectious disease were obtained by local health departments and forwarded to the State Health Department were continued independently of the study. During most of the study period, the only CNS disease entities officially reportable to health departments and which were very likely to be observed in the hospital study were "acute encephalitis," "acute anterior poliomyelitis" (designated as paralytic or non-paralytic), and "meningococcal meningitis." "Meningitis, aseptic or viral" was added to the list of reportable diseases just prior to the close of the study in 1958. Official morbidity reports were initiated by various individuals, usually attending physicians, hospital staff personnel or local health department personnel, and subject to verification by the local health officer, were forwarded to the State Health Department. Rosters of the cases observed in each hospital were subsequently checked against the official morbidity reports received by the State Health Department.

Isolation and identification of viruses. Stool suspensions were inoculated into monolayer cultures of monkey kidney cells which were observed for cytopathic effects. The viruses recovered were typed either in monolayer cultures of monkey kidney cells or colorimetrically[12] in cultures of human amnion (FL) cells against immune sera to the polioviruses, Coxsackie virus types A9 and B1 through B5, and available ECHO virus types.

Serologic tests. Acute- and convalescent-phase sera were tested for complement-fixing (CF) antibody to the viruses of WEE, SLE, mumps, herpes simplex, and poliomyelitis, by the methods previously described.[14, 13] Sera from patients yielding a Coxsackie virus, and also from a selected group of patients with negative stools, were tested for neutralizing antibody against Coxsackie viruses B1–B5 by an in vitro colorimetric method.[13]

RESULTS

Reported incidence of encephalitis. The total annual and quarterly reported incidence of acute encephalitis from the entire state during 1956,

TABLE 1

Reported cases of encephalitis by etiology and calendar quarter in California, 1956–1958

Year and quarter of onset	No. cases	Etiologic category %*					
		Total	WEE†	SLE‡	Mumps	Other§	Undetermined
1956	*558*	*100*	*3*	*1*	*45*	*16*	*35*
Jan.–Mar.	126	100	0	0	63	16	21
Apr.–June	178	100	1	0	59	19	21
July–Sept.	171	100	8	4	25	12	51
Oct.–Dec.	83	100	0	1	31	14	53
1957	*511*	*100*	*1*	*5*	*36*	*27*	*31*
Jan.–Mar.	127	100	1	0	40	45	14
Apr.–June	186	100	0	0	42	32	25
July–Sept.	157	100	1	14	27	13	44
Oct.–Dec.	41	100	0	2	32	5	61
1958	*526*	*100*	*7*	*3*	*29*	*20*	*41*
Jan.–Mar.	96	100	0	0	38	35	27
Apr.–June	150	100	5	1	43	30	21
July–Sept.	221	100	13	5	19	7	55
Oct.–Dec.	59	100	2	5	12	20	61

* Per cents are rounded independently and may not add to totals.

† WEE = Western equine encephalomyelitis.

‡ SLE = St. Louis encephalitis.

§ Other includes measles, chicken-pox, rubella, influenza, herpes simplex, and post-vaccinal encephalitides.

1957 and 1958 is shown in Table 1, according to the etiologic classification recorded in the morbidity records of the State Health Department. The etiologic classes shown in Table 1 reflect the results of serologic tests for WEE, SLE, mumps, and herpes simplex provided by the regular diagnostic services of the Viral and Rickettsial Disease Laboratory but do not take into account supplementary laboratory studies for the detection of enteroviruses. A diagnosis of WEE or SLE was recorded only if infection was established by isolation of the virus, by demonstration of significant antibody rises or, in a few instances, by the presence of elevated convalescent antibody titers (CF $\geq$ 1:16). During this 3-year period, as in previous years,[3, 4] the mumps virus was by far the most commonly implicated etiologic agent in cases reported as encephalitis from the state as a whole, accounting for some 40 to 60% of the cases during the late winter and spring, and exceeding throughout the year the number of cases due to other etiologic agents. The two arthropod-borne viruses, limited in both seasonal and geographic occurrence, accounted for only 4 to 10% of the reported annual incidence, but during the summer months, WEE and SLE accounted for 12 to 18% of the reported cases. Of the two, WEE was more frequently encountered in 1956 and 1958, and SLE in 1957. Encephalitic illnesses attributed to various other infections accounted for 16 to 27% of the annual total, and these, as was true for mumps, had a greater incidence during the late winter and spring quarters. In roughly one-third of the cases each year the etiology was recorded as undetermined. The proportion of such cases tended to increase during the summer and fall quarters, often comprising half or more of the reported incidence. Although the "undetermined group" includes some cases in which WEE, SLE or mumps cannot be excluded, *e.g.*, those with low stationary antibody levels or those in which serologic studies were not done, over half of the patients in this group had completely negative

TABLE 2

Diagnoses from Health Department morbidity reports compared with clinical classification made from medical records of cases of suspected central nervous system disease observed in four selected study areas in the California Central Valley, 1956–1958

Morbidity report diagnosis	Clinical classification from medical records							
	Total cases	Enceph.	Doubtful enceph.*	Aseptic meningitis	Paralytic polio	Purulent meningitis	Other CNS disease	No CNS signs
Total cases	*335*	*53*	*39*	*130*	*48*	*15*	*20*	*30*
1956	150	17	17	63	33	4	5	11
1957	84	14	13	29	6	5	6	11
1958	101	22	9	38	9	6	9	8
Encephalitis	*153*	*48*	*33*	*59*	*2*	*3*	*6*	*2*
1956	46	15	12	17	—	—	2	—
1957	39	12	13	11	—	—	2	1
1958	68	21	8	31	2	3	2	1
Nonparalytic polio	*51*	—	*2*	*48*	*1*	—	—	—
1956	40	—	2	37	1	—	—	—
1957	11	—	—	11	—	—	—	—
1958	—	—	—	—	—	—	—	—
Paralytic polio	*47*	—	—	—	*45*	—	*2*	—
1956	32	—	—	—	32	—	—	—
1957	8	—	—	—	6	—	2	—
1958	7	—	—	—	7	—	—	—
Other, or not reported	*84*	*5*	*4*	*23*	—	*12*	*12*	*28*
1956	32	2	3	9	—	4	3	11
1957	26	2	—	7	—	5	2	10
1958	26	1	1	7	—	3	7	7

* Aseptic meningitis syndrome with doubtful encephalitic involvement.

serologic tests for these viruses. These data serve to illustrate the extent of the occurrence of reported encephalitic illnesses of obscure etiology in California, which prompted further inquiry.

Cases of CNS disease observed in four health jurisdictions in the Central Valley. In the present study, a total of 335 patients (150 patients in 1956, 84 in 1957, and 101 in 1958) with suspected CNS disease was observed from the four health jurisdictions. The clinical terminology used in assigning diagnoses varied appreciably among attending physicians, even within the same hospital. Thus, the same clinical syndrome might be called "viral meningitis," "meningoencephalitis," "nonparalytic poliomyelitis," or "encephalitis, possible polio" depending on personal factors. To obviate this variation in terminology, the medical records of all patients were reviewed by one investigator (RM) who applied uniform criteria to classify the illness of each patient. This was done without reference to the results of viral studies. The results of this classification are shown in Table 2. In 53 cases the illness was classified as unequivocal encephalitis characterized by fever, headache, signs of meningeal irritation and one or more of the following signs of a frank encephalitis: lethargy or stupor, mental confusion, convulsions, tremor, muscular twitchings, ataxia, nystagmus, or disturbance of speech. In 130 cases, the illness was classified as aseptic meningitis; these patients had fever, headache, signs of meningeal irritation, and elevated leukocyte counts of the spinal fluid, but none of the aforementioned signs of frank encephalitis. In an additional 39 cases with similar manifestations of aseptic meningitis, there were borderline or equivocal signs of encephalitis such as moderate drowsiness or a single convulsion accompanying fever in a child. These were classified as "doubtful encephalitis." Forty-eight cases were classified as paralytic poliomyelitis on the basis of characteristic localized muscle weakness or paralysis. Fifteen cases were readily classified as purulent meningitis on the basis of highly elevated leukocyte counts in the spinal fluid, with polymorphonuclear cells predominating; the diagnosis was supported in most instances by cultural evidence of a bacterial infection. Twenty cases, representing a diverse group of neurologic disorders, *e.g.*, Guillain-Barré syndrome, polyneuritis, epilepsy, tumors or vascular lesions, were grouped under the classification "other CNS diseases." In 30 cases, the initial suspicion of a possible CNS involvement was not borne out on further observation and no definitive signs of CNS involvement were recorded.

A comparison of this clinical classification with the diagnoses given on official morbidity reports received by the State Health Department is presented in Table 2. Although 153 cases in the hospital study series were reported as encephalitis, in our classification only 48 of these were considered to be unequivocal cases of encephalitis and 33 were considered to have doubtful encephalitic signs; the remainder were scattered in other categories. Official morbidity reports of paralytic poliomyelitis were in close agreement with our classification. Cases reported as nonparalytic poliomyelitis coincided well with our classification of aseptic meningitis. Among the cases in the hospital series not officially reported to the State Health Department were 9 classified as encephalitis or doubtful encephalitis, and 23 classified as aseptic meningitis.

Annual variation in types of CNS disease. Variations in the occurrence of the different clinical types of CNS disease during the three summers are summarized in Table 3. Paralytic poliomyelitis, which accounted for 24% of the cases in 1956 declined to 10% or less in 1957 and 1958. The decline in poliomyelitis was largely responsible for the smaller total number of cases observed in 1957 and 1958, and led to a relative

TABLE 3

Per cent distribution of clinical syndromes by year of onset

Clinical syndrome	Per cent* of cases by year			
	1956–1958	1956	1957	1958
Total	100	100	100	100
Encephalitis	17	12	19	24
Doubtful encephalitis	13	12	18	10
Aseptic meningitis	43	45	40	41
Paralytic polio	16	24	8	10
Purulent meningitis	5	3	7	6
Other CNS diseases	7	4	8	10
No. cases	335	150	84	101
(No CNS signs†)	(30)	(11)	(11)	(8)

* Per cents are founded independently and may not add to total.

† Excluded from per cent distribution.

increase in the proportion of encephalitis cases, from 12% in 1956 to 24% in 1958. In each year, cases of aseptic meningitis comprised some 40 to 45% of the illnesses. Together with cases of doubtful or borderline encephalitis, aseptic meningitis accounted for 50 to 60% of the CNS diseases observed each summer.

Laboratory evidence of etiology. Satisfactory specimens for viral studies were obtained from 310 of the 335 patients. The results of viral studies are summarized in Table 4 according to the clinical classification described above. A detailed breakdown of the specific immunotypes of viruses associated each year with illnesses classified as encephalitis, doubtful encephalitis or aseptic meningitis is given in Table 5. Of 48 patients classified as frank encephalitis, serologic evidence of an arthropod-borne virus infection was elicited in 21, viz., 17 WEE (35%) and 4 SLE (8%). Coxsackie B4 and B5 viruses were recovered from the stools of two infants in the group of WEE cases. Both exhibited significant rises in CF antibody to WEE virus during the course of illness and also had elevated titers of homotypic neutralizing antibody to the Coxsackie virus recovered. In 6 of the encephalitis cases (13%) a group B Coxsackie virus was the only virus implicated, and in 1 case an unidentified enterovirus was the only agent. Mumps virus infection was implicated in only one (2%) of the cases of frank encephalitis and a significant rise in antibody to herpes simplex virus was elicited in two cases (4%). In 18 cases (38%) one of which was considered to be a post-measles encephalitis, no viral infection was detected by laboratory tests.

WEE and SLE viruses were encountered among patients classified as doubtful encephalitis or aseptic meningitis, but much less often than in the frank encephalitis cases, whereas mumps virus infection was notably more often associated with illnesses in the doubtful encephalitis group. Group B Coxsackie viruses were encountered with about equal frequency in each clinical category, whereas poliovirus, ECHO and unidentified enteroviruses were associated more often with cases of aseptic meningitis. In nearly half

TABLE 4

Laboratory findings in cases of suspected central nervous system (CNS) disease observed in California Central Valley, summers of 1956–1958

Clinical classification	No. of cases†	Laboratory diagnosis: %*								Undetermined	
		Total	WEE	SLE	Mumps	Herpes simplex	Coxsackie B2-B5	ECHO or unident.	Polio	Stools and sera tested	Only sera tested
Encephalitis	48	100	35‡	8	2	4	13	2	0	29§	6
Doubtful encephalitis	38	100	8	3	24	0	11	5	5	32	13
Aseptic meningitis	123	100	4	0	7‖	0	16	10	16	28	18
Paralytic polio	48	100	0	2	0	0	0	0	85	8	4
Purulent meningitis	10	(100)	(0)	(0)	(0)	(0)	(0)	(20)	(0)	(60)	(20)
Other CNS disease	17	(100)	(0)	(0)	(0)	(0)	(6)	(0)	(6)	(71)	(18)
No CNS signs	26	100	0	0	4	0	10	0	4	42	50
Total	310	100	8	2	6	1	10	5	21	30	16
Cases officially reported as "encephalitis"	147	100	14‡	4	11‖	1	14	7	7	27§	14

* Per cents are rounded independently and may not add to totals; figures in parentheses indicate per cents based on less than 20 cases.

† Omits 25 cases included in Tables 2 and 3 in which specimens were inadequate for laboratory study.

‡ Includes 2 infants who had 8-fold or greater rises in antibody titer to WEE virus and whose stools yielded Coxsackie virus, types B4 and B5 respectively.

§ Includes 1 case of post-measles encephalitis.

‖ ECHO virus, type 10, was isolated from a stool specimen of one patient.

of the patients classified as doubtful encephalitis or aseptic meningitis, no viral infection was established; however, stool specimens were not submitted from a number of these patients (Table 4).

Of the 48 patients clinically classified as paralytic poliomyelitis, poliovirus infection was confirmed by virus isolation and/or a significant antibody rise in 41 cases (85%). In 6 cases, poliovirus was not recovered and the results of serologic tests were inconclusive, but were compatible with current or recent poliovirus infection. In one case, that of a 50-year-old man, considered clinically to have fulminating bulbar poliomyelitis, SLE virus was recovered from brain material taken at autopsy and samples of brain, spinal cord and colon failed to yield poliovirus.

In two instances an ECHO virus was recovered from the stool of infants with purulent meningitis. In one of these cases, a 22-month-old boy, *Hemophilus influenzae* was cultured from the spinal fluid and ECHO 24 virus was recovered from a stool taken 9 days after onset. The other case was

TABLE 5

Laboratory diagnosis in cases of clinical encephalitis or aseptic meningitis observed in 4 study areas in California Central Valley 1956–1958

Laboratory diagnosis	All cases	Year of onset											
		1956–1958			1956			1957			1958		
		Enceph.	Doubt. enceph.	Aseptic menin.	Enceph.	Doubt. enceph.	Aseptic menin.	Enceph.	Doubt. enceph.	Aseptic menin.	Enceph.	Doubt. enceph.	Aseptic menin.
WEE	25	17	3	5	3	2	3	1*	—	—	13†	1	2
SLE	5	4	1	—	1	—	—	3	—	—	—	1	—
Mumps	19	1	9	9	1	4	5‡	—	4	3	—	1	1
Herpes simplex	2	2	—	—	—	—	—	1	—	—	1	—	—
Coxsackie													
B2	2	1	—	1	1	—	—	—	—	—	—	—	1
B3	2	—	1	1	—	1	1	—	—	—	—	—	—
B4	10	3	2	5	—	—	—	3	2	4	—	—	1
B5	14	1	1	12	—	1	9	—	—	1	1	—	2
No isol., neut. antibody rise, B5	2	1	—	1	—	—	—	—	—	—	1	—	1
ECHO													
6	4	—	1	3	—	—	—	—	—	—	—	1	3
9	5	—	1	4	—	—	—	—	—	1	—	1	3
10	1	—	—	1	—	—	—	—	—	—	—	—	1
19	1	—	—	1	—	—	—	—	—	—	—	—	1
Unident.	4	1	—	3	—	—	2	—	—	1	1	—	—
Polio													
1	9	—	—	9	—	—	9	—	—	—	—	—	—
3	3	—	1	2	—	—	2	—	1	—	—	—	—
No isol., CF antibody rise	10	—	1	9	—	—	3	—	1	1	—	—	5
Etiology undetermined													
Stools and bloods	61	14	12	35	6	5	16	4	4	11	4§	3	8
Serology only	30	3	5	22	3	3	9	—	1	4	—	1	9‖
Total	209	48	38	123	15	16	59	12	13	26	21	9	38

* Coxsackie B5 virus isolated from 1 of 4 stools.
† Coxsackie B4 virus isolated from 3 stools of 1 patient.
‡ ECHO 10 virus isolated from 1 patient.
§ One patient with post-measles encephalitis.
‖ One patient with probable coccidiodomycosis.

that of a 14-day-old infant girl, with a spinal fluid leukocyte count of 2,500 cells per cmm, 97% polymorphonuclear, and negative smears and bacteriologic cultures of the spinal fluid. ECHO virus type 9 was recovered from the feces 10 days after onset. While it is uncertain that ECHO 9 virus was causally related to the illness of this patient, it is noteworthy that spinal fluid cell counts in excess of 2,000 cells per cmm have been observed in ECHO 9 virus infections.[16]

Of the 310 laboratory-studied cases (Table 4), 147 were officially reported as encephalitis, although only one-third of these met the criteria of a frank encephalitis in our classification. In this group of 147 reported cases, illnesses due to the arthropod-borne viruses were proportionately much less frequent, and those attributed to the mumps virus were more frequent than in the group of cases classified as frank encephalitis.

The array of viruses associated with encephalitis and aseptic meningitis during each of three consecutive summers is shown in Table 5. After 2 years of low incidence, there was an apparent increase of WEE in 1958, when the WEE virus accounted for 13 of the 21 cases (62%) of frank encephalitis. One or more well defined encephalitic illnesses were associated with a Coxsackie group B virus each summer; the immunotypes implicated were type B2 in 1956, B4 in 1957, and B5 in 1958. In the latter 2 years, the same types (B4 and B5, respectively) of Coxsackie virus were also widely associated with aseptic meningitis in other areas of the state as well as in the Central Valley. ECHO viruses were infrequently encountered in cases of CNS disease in the Central Valley in 1956 and 1957, but ECHO types 6, 9, 10 (Reovirus) and 19 were recovered from patients with aseptic meningitis or doubtful encephalitis in 1958. Type 9 was the most frequently encountered ECHO virus in other areas of the state in 1958.

Dual infections. In three instances, evidence of infection with two different viruses was elicited. Two cases, as already mentioned above, were infants with clinical encephalitis, both of whom had significant rises in CF antibody titer to WEE virus in the convalescent-phase serum specimens at 15 and 32 days, respectively, after the onset of illness. From one of these infants, Coxsackie B5 virus was recovered from a single stool taken 11 days after onset of illness and homotypic neutralizing antibody was present at a titer of 1:128 in both the early and late sera. The other infant yielded Coxsackie B4 virus from three stools, collected 2, 3, and 5 days after onset, and the neutralizing antibody to this virus increased from 1:32 to 1:128 during the illness. Although either the WEE virus or the Coxsackie virus, or both viruses may have contributed to the illness of these infants, we have included them with the

TABLE 6

Clinical spectrum of central nervous system diseases associated with certain etiologic agents in California Central Valley 1956–1958

Clinical classification	Total cases	Per cent* distribution of cases in each etiologic category							
		WEE	SLE	Mumps	Herpes simplex	Cox. B	ECHO	Polio	Etiology undetermined
Total, %	100	100	(100)†	100	(100)	100	(100)	100	100
Encephalitis	15	68	(67)	5	(100)	19	(6)	0	12
Doubtful encephalitis	12	12	(17)	45	(0)	13	(12)	3	12
Aseptic meningitis	40	20	(0)	45	(0)	65	(71)	31	40
Paralytic polio	15	0	(17)	0	(0)	0	(0)	63	4
Purulent meningitis	3	0	(0)	0	(0)	0	(12)	0	6
Other CNS	5	0	(0)	0	(0)	3	(0)	2	10
No CNS	8	0	(0)	5	(0)	0	(0)	2	17
No. of cases	310	25‡	6	20	2	31	17	65	144

* Per cents are rounded independently and may not add to 100.

† Parentheses indicate percentage based on less than 20 cases.

‡ Includes 2 cases in which a Coxsackie group B virus was recovered from the stools; these 2 cases are excluded from the column of Coxsackie B infections.

TABLE 7

Cases of meningoencephalitic illness associated with Coxsackie group B viruses

Case no.	Age, sex	Date onset	Clinical classification, major signs and symptoms	Spinal fluid cells	Coxsackie virus isol.		Antibody determinations									
					Day	Type	Day	Coxsackie neut.					CF			
								B1	B2	B3	B4	B5	WEE	SLE	Mumps
1	5 wk F	7/7/56	Severe encephalitis. Fever, convulsions, stupor, muscular twitchings of face and eyelids; recovery in 2 weeks	122 WBC 60% lymphs	5	B2	5	<8	128	<8	<8	<8			
					11	B2	20	<16	1024	<16	<16	<16	<8	<8	<8
2	25 yr M	7/27/57	Severe encephalitis. Fever, headache, stiff neck, convulsions, emotional lability; residual writing aphasia for 2 weeks	20 WBC 80% lymphs	4	B4	2	<32	<32	<32	<32	<32			
					6	B4	16	<8	<8	<8	256	<8	<8	<8	<8
					12	B4	58	<8	<8	<8	64	<8	<8	<8	<8
3	5 yr F	7/29/57	Moderately severe encephalitis. Fever, stiff neck, headache, convulsions, drowsiness, mental confusion. Hospitalized 10 days	660 WBC 65% lymphs	3	Neg.	3	<8	256	<8	<8	<8			
					8	B4	24	<8	512	<8	<8	<8	<8	<8	<8
4	6 wk M	8/6/57	Moderately severe encephalitis. Fever, convulsions, lethargy, tremors, muscular twitchings; defervescence after 5 days	374 WBC 80% polys.	8	B4	No sera								
					17	B4									
5	3 yr M	7/17/58	Moderately severe encephalitis. Fever, stiff neck, headache, lethargy, muscular twitchings, irritability.	2300 WBC 64% lymphs	No stools		1	<8	<8	<8	<8	32			
							13	<8	<8	<8	<8	256	<8	<8	NS
6	4 mo F	7/24/58	Moderately severe encephalitis. Fever, stiff neck, convulsions lethargy, tremors, muscular twitching.	397 WBC 80% polys.	2	B5	No sera								
					3	Neg.									
					4	Neg.									
7	9 yr M	8/25/56	Doubtful encephalitis. Fever, headache, stiff neck, moderate drowsiness.	1496 WBC 70% polys.	7	B3	4	<8	<8	128	<8	<8			
							31	<16	<16	256	<16	<16	<8	<8	<8
8	14 yr F	9/22/56	Doubtful encephalitis. Fever, headache, stiff neck, moderate drowsiness.	35 WBC 92% lymphs	5	B5	5	<8	16	64	32	8			
							17	32	128	1024	256	256	<8	<8	<8
9	23 yr M	8/11/57	Doubtful encephalitis. Fever, headache, stiff neck, drowsiness.	202 WBC 83% lymphs	5	B4	2	32	<8	<8	16	<8			16
					7	Neg.	46	64	<8	<8	64	8	<8	<8	16
10	5 yr F	8/13/57	Doubtful encephalitis. Fever, headache, vomiting, stiff neck, drowsiness, epigastric pain.	800 WBC 75% polys.	3	B4	3	<8	1024	<8	64	<8			
					10	B4	104	<8	1024	<8	128	<8	<8	<8	<8

WEE cases in the accompanying tables. In a third case of apparent dual infection, ECHO virus type 10 was recovered from the stool of a 10-year-old boy who was clinically considered to have mumps meningitis and who exhibited a rise in mumps antibody titer from 1:16 to 1:64. This case is tabulated with the mumps virus infections.

Clinical spectrum of CNS syndrome associated with certain viruses. Since this study embraced a wide spectrum of acute CNS diseases, it was possible to compare the relative frequency with which particular clinical syndromes were associated with certain viruses, as shown in Table 6. In general, the spectrum of illness related to each virus was in keeping with previously established etiologic relationships. Thus nearly 70% of the illnesses associated with WEE or SLE infections were classified as frank encephalitis; 65% of the poliovirus infections were manifested clinically as paralytic poliomyelitis; and 65 to 70% of the illnesses associated with Coxsackie and ECHO virus infections were classified as aseptic meningitis having no paralytic or encephalitic involvement. The mumps virus infections were divided evenly between the clinical classifications of aseptic meningitis and doubtful encephalitis; only one patient with mumps virus infection (5%) was classified as frank encephalitis. Coxsackie virus infections were associated with an unequivocal encephalitic illness more frequently than were mumps virus infections. Except for one patient who yielded an unidentified cytopathic agent, presumably an ECHO virus, frank encephalitic illnesses were not linked with ECHO or poliovirus infections in the course of this study.

The clinical and laboratory findings in each of the cases of frank or borderline encephalitis associated with a Coxsackie virus infection are summarized in Table 7. Cases 1 to 6 are those shown in previous tables as "encephalitis"; cases 7 to 10 are those classified as "doubtful encephalitis." In two cases (4 and 6) sera were not submitted and Coxsackie virus was implicated only by the recovery of virus from the stools. In the remaining cases, the results of neutralizing antibody determinations provided additional evidence suggesting that Coxsackie virus infection was temporally related to the current illness. In case 3, the isolate was typed as B4, but an elevated titer of neutralizing antibody was encountered only to type B2; however, we have previously noted[17] high heterotypic antibody titers and titer rises to type B2 virus among patients excreting type B4. Severe encephalitic involvement occurred primarily in infants and young children but one case (2) occurred in a 25-year-old man in the course of infection with Coxsackie B4. Four cases (7 to 10), ranging from

TABLE 8

Age and sex distribution of cases of viral CNS disease in the California Central Valley 1956–1958

Age group	Total cases	Etiologic classification									
		WEE	SLE	Mumps	Herpes simplex	Cox. B	ECHO or unident.	Polio	Undetermined		
									Encephalitis	Doubtful encephalitis	Aseptic meningitis
<1	25	12	—	—	—	5	4	2	—	—	2
1–4	40	1	1	5	1	2	4	17	1	2	6
5–9	59	5	—	5	—	7	3	16	2	6	15
10–14	29	1	—	4	1	4	2	6	3	—	8
15–19	21	—	1	1	—	3	—	8	—	5	3
20–29	44	1	1	4	—	9	4	9	4	1	11
30–39	24	2	—	1	—	1	—	5	4	2	9
40+	15	3	3	—	—	—	—	2	3	1	3
Total	257	25	6	20	2	31	17	65	17	17	57
Male	152	15	5	14	2	16	8	37	11	8	36
Female	105	10	1	6	—	15	9	28	6	9	21

5 to 23 years of age, were classified as "doubtful encephalitis" on the basis of moderate drowsiness; otherwise, these cases were indistinguishable from a benign aseptic meningitis syndrome.

Age and sex—influence of age on clinical syndrome. The age and sex of the patients in relation to the etiologic findings are shown in Table 8.

Nearly half of the WEE virus infections were in infants under 1 year of age, in keeping with previous observations in California.[2-5] None of the other agents showed a similar marked predilection for any one age group, although poliovirus, mumps and Coxsackie virus infections occurred most frequently in children under age 15. Cases of encephalitis and aseptic meningitis of undetermined etiology were widely scattered in all age groups but were somewhat more frequent in school-age children and adults than in the very young. Males predominated in all etiologic categories except ECHO virus infections. As noted in previous studies in California, the male-female ratio was particularly high in the arthropod-borne encephalitides, encephalitis of undetermined etiology,[2, 5] and mumps CNS disease.[18]

A further breakdown of the age distribution of patients with Coxsackie virus infection according to the type of CNS disease revealed a rather marked grouping of the frank encephalitic illnesses in infants and young children approaching the age pattern seen in WEE (Table 9). Thus five of the six encephalitic illnesses associated with a Coxsackie virus infection were in children under age 6, and three of these were infants under 6 months of age. Stated in another way, half of the Coxsackie virus infections encountered in small children were associated with a frank encephalitic illness. With increasing age encephalitic manifestations were less frequently observed, and a benign aseptic meningitis syndrome was the predominant illness. A similar tendency toward a milder illness and less definite encephalitic manifestations with increasing age was also seen in WEE infections, *e.g.*, five of the twelve illnesses associated with WEE virus in patients over 5 years of age were classified as an aseptic meningitis syndrome. The changes in clinical manifestations with increasing age noted here are in conformity with previous observations of the more serious character of illness in WEE[19–21] and Coxsackie virus disease[11, 12] occurring in infancy.

TABLE 9

Acute CNS diseases associated with Coxsackie group B and Western equine encephalitis virus according to age

Age group	Laboratory diagnosis and clinical syndrome					
	Coxsackie B			WEE		
	Encephalitis	Doubtful encephalitis	Aseptic meningitis	Encephalitis	Doubtful encephalitis	Aseptic meningitis
<1	3	—	2	11*	1	—
1–4	1	—	1	1	—	—
5–9	1	2	4	3	—	2
10–14	—	1	3	—	—	1
15–19	—	—	3	—	—	—
20–29	1	1	6	—	1	—
30+	—	—	1	2	1	2
Total	6	4	20	17	3	5

* Includes two infants who had significant rise in antibody titer to WEE virus and whose stools yielded Coxsackie virus, type B4 and B5, respectively.

COMMENT AND SUMMARY

The results of this inquiry indicated that illnesses reported as encephalitis to health departments in the Central Valley of California covered a broad spectrum of syndromes ranging from frank severe encephalitis to mild meningitic illness with no definite signs of cerebral involvement. Of 153 illnesses observed during three consecutive summers and recorded as cases of encephalitis in official morbidity records, unequivocal encephalitic manifestations were noted in approximately 30%, and borderline encephalitic signs in an additional 22%. In the entire group of reported encephalitis cases, group B Coxsackie virus and WEE virus infections were encountered with equal frequency, although each virus was associated with only 14% of the cases. Mumps, ECHO, SLE, and herpes simplex viruses, in declining order of frequency, were each associated with a lesser number of illnesses, but in 40% of the cases no viral infection was established. When only the illnesses classified unequivocally as encephalitis were considered, however, the proportion attributed to WEE virus increased to 35%; Coxsackie virus infection was associated with 13%, and other virus infections were encountered in less than 10% of the cases. In the cases of doubtful encephalitis, the mumps virus

was implicated most frequently (25%) followed by Coxsackie B virus (11%), WEE virus (8%), and SLE virus (3%). In illnesses classified as aseptic meningitis with no signs of encephalitic involvement, group B Coxsackie and polioviruses were the agents most frequently encountered (each in 16%) followed by ECHO (10%), mumps (7%) and WEE (4%). In each clinical category, no viral infection was established in 35 to 45% of the cases. There was appreciable variation in these proportions from year to year, but in each year the arthropod-borne viruses, WEE and SLE together, were implicated most often in cases classified as frank encephalitis, and Coxsackie group B viruses equaled or exceeded the mumps virus in such illnesses. Of the other enteroviruses, an ECHO virus (type undetermined) was associated in only one instance, and poliovirus not at all, with frank encephalitis. Inasmuch as the role of group B Coxsackie viruses as causal agents of aseptic meningitis is well established,[7,9] the association of these viruses with 12 to 19% of such illnesses each year is believed to be a reasonable approximation of their relative etiologic importance in the endemic occurrence of this syndrome in the particular localities and seasons represented in this study.

While the frank encephalitic illness observed in association with group B Coxsackie viruses in this study could reflect the occurrence of coincidental infections in persons whose illness was due to other unrecognized causes, the probability of an etiologic association may be supported on several grounds: (1) the frequency of association of group B Coxsackie viruses with encephalitic illness equaled or exceeded that of other commonly recognized encephalitogenic viruses including mumps and herpes simplex, and was exceeded only by WEE virus; (2) the consistency of such association over three consecutive summer periods; (3) the lack of a similar association of poliovirus or ECHO viruses with encephalitis, although both of the latter were associated with other illnesses in the areas under study and could have been present in coincidental alimentary infections; (4) the occurrence of rises in titer of neutralizing antibody to Coxsackie virus, indicating a temporal relationship of the infection to the encephalitic illness; and (5) the concentration in infants and young children of encephalitic illness associated with Coxsackie viruses.

It appears reasonable to conclude that perhaps 10 to 15% or more of the incidence of summer illnesses reported as encephalitis from the California Central Valley in recent years may have been attributable to Coxsackie group B viruses. Although the majority of the reported cases of encephalitis found to be associated with a Coxsackie virus infection could more appropriately be classified as an aseptic meningitis syndrome, group B Coxsackie viruses may also have accounted for possibly 10 to 15% of the frank encephalitic illnesses. Poliovirus and ECHO viruses were also implicated in about 15% of the cases reported as encephalitis, but on review these cases were almost uniformly classified clinically as aseptic meningitis or doubtful encephalitis. Despite the implication of enteroviruses in over one-fourth of the cases of reported encephalitis, there still remained roughly one-fourth of the cases in which both virus isolation attempts and serologic tests failed to establish a possible viral etiology.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Nathalie Schmidt, Miss Anna Wiener, Mr. Tak Shinomoto and Mrs. Florence Jensen for their valuable laboratory contributions to this study, and to Mr. Ray Guenther for assistance in analysis of the data. Thanks are also due the health officers, and their staffs, of the Kern, Fresno, San Joaquin and Sutter-Yuba County Health Departments for their generous co-operation in obtaining laboratory specimens and clinical data.

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COMPARATIVE SENSITIVITY OF FOUR HOST SYSTEMS FOR THE ISOLATION OF CERTAIN ARTHROPOD-BORNE VIRUSES FROM MOSQUITOES*

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Recent reports have indicated that certain tissue culture systems may be used for the isolation and study of many of the arthropod-borne (arbor) viruses. Thus, it has been found that a number of these viruses are consistently cytopathogenic in hamster kidney cells[1, 2] and that some of the group produce specific cytopathic effects in cultures of chick embryo fibroblasts.[3-5]

Before converting to the exclusive use of tissue culture systems for the isolation and study of arthropod-borne viruses it would seem desirable to have more extensive information on the sensitivity of these systems for the primary isolation of agents from field materials as compared to the sensitivity of embryonated eggs and mice.

For the past several years, this laboratory has examined as part of an Encephalitis Surveillance Program, large numbers of mosquito pools for the presence of viral agents; the agents recovered, through the use of embryonated hens' eggs and adult mice, have been the viruses of Western equine encephalomyelitis (WEE), St. Louis encephalitis (SLE), and the recently described Turlock virus.[6]

During the 1958 study year (May 1 to October 1), all mosquito pools examined in this laboratory were inoculated into mice and embryonated eggs and, additionally, into cultures of hamster kidney cells and chick embryo fibroblasts.

This report compares the sensitivity of the four host systems for the isolation of three arthropod-borne viruses, and discusses the rapidity and ease of identification of the agents in the various host systems.

MATERIALS AND METHODS

Collection of mosquito pools. For the 1958 study season, most of the mosquitoes examined were collected in Sutter County, California, and the predominant mosquito in the collections was *Culex tarsalis*. Procedures for collecting the mosquitoes and preparing the pools for shipment to the laboratory have been described in a recent publication from this laboratory.[6]

Preparation of mosquito pools for inoculation into the four host systems. Each pool† of mosquitoes was triturated individually in a mortar and suspended in a diluent consisting of buffered saline‡ with an 0.75% concentration of bovine albumin; at the time of use, penicillin and streptomycin were added to this diluent to give a final concentration of 500 units and 500 μg respectively per ml. The mosquito pool-antibiotic mixtures were incubated for 30 minutes at 4°C, then centrifuged in an angle rotor (Spinco) for 15 minutes at 10,000 rpm. The supernatant fluids were removed, care being taken not to disturb the sediment and the pellicle of fatty material, and the fluids were then divided into the following three portions: (1) a sample of 0.4 ml for mouse inoculation; (2) a portion of 0.9 ml, to which was added 0.1 ml of an antibiotic solution containing 10,000 units of penicillin and 50,000 μg of streptomycin per ml for inoculation into embryonated hens' eggs; and (3) an aliquot of 0.9 ml to which was added 0.1 ml of the antibiotic solution just mentioned for inoculation into hamster kidney and chick embryo tissue cultures.

Each mosquito pool suspension was inoculated into all four host systems in parallel.

Inoculation of mice. Each mosquito pool suspension was inoculated intracerebrally in 0.03 ml amounts into eight albino Swiss mice 3 to 4 weeks old. The animals were kept under observation for 14 days for signs of illness.

Inoculation of embryonated eggs. The antibiotic-

* This work was supported in part by a grant (E-1475) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

† Pools usually contained 50 mosquitoes. However, at the beginning and toward the end of the season, when population densities were low, the pools were comprised of lesser, and variable, numbers. All pools, irrespective of size, were triturated in 4 ml of diluent.

‡ The buffered saline solution had the following composition: NaCl, 3.4 g; KH_2PO_4, 2.5 g; $Na_2HPO_4 \cdot 12H_2O$, 28.2 g; and distilled water to 1,000 ml.

treated mosquito pools were inoculated into the amniotic sac of 10 to 11-day-old embryonated eggs, each suspension being inoculated into eight eggs in 0.1 ml volumes. Inoculated eggs were candled daily for 7 days.

Inoculation of hamster kidney and chick embryo tissue cultures. Monolayer cultures of the two cell types, prepared as described below, were inoculated with 0.1 ml of the antibiotic-treated mosquito pool suspensions. Each pool was inoculated into four culture tubes of each cell type. All mosquito pools were routinely passaged three times in each cell type before they were considered negative, and the cultures of each passage were examined over a 7-day period for evidence of cytopathogenesis.

Neutralization tests in mice. All neutralization tests were performed in mice 1 to 3 days old. The procedures for the identification of Turlock and SLE viruses by mouse neutralization tests have been described elsewhere.[6,7]

Tissue culture neutralization tests for SLE. In addition to being identified in mouse neutralization tests, all SLE isolates recovered from hamster kidney tissue cultures were identified in tube cultures of this cell type. For this procedure, 0.5 ml volumes of 1×10^{-2} and 1×10^{-3} dilutions of the isolates were mixed with 0.5 ml of a 1:5 dilution of SLE immune rabbit serum. The serum-virus mixtures were incubated for 1 hour at 4°C, after which 0.2 ml of each serum-virus mixture was inoculated into four tubes of hamster kidney cells. Appropriate controls on each virus dilution in the absence of immune serum were included. The tests were examined over a period of 7 days for specific inhibition of cytopathogenesis by the immune serum.

Identification of isolates by the complement fixation technique. Isolates of WEE virus recovered in embryonated eggs were identified by the complement fixation procedure previously described from this laboratory,[7] and all WEE isolates recovered in the tissue culture systems were also identified by complement fixation, employing the infected tissue culture fluids as antigens. Turlock viruses isolated in chick embryo tissue cultures, in addition to being identified in mouse neutralization tests, were identified by complement fixation tests on the infected fluids; those strains isolated in embryonated eggs were passed to chick embryo tissue cultures and the infected culture fluids were subsequently employed as complement-fixing antigens for identification purposes.

Preparation of chick embryo tissue culture. The torsos of 9-day-old chick embryos were rinsed three times in Hanks' balanced salt solution (BSS), and after the third rinse they were minced into pieces approximately 3 mm in diameter. The minced tissue was then rinsed three times in Hanks's BSS and passed through a 50-ml syringe (without a needle) into a heavy-walled flask, where the mince was rinsed two more times with Hanks' BSS. An appropriate volume§ of an 0.25% solution of trypsin in Hanks' BSS was added to the tissue mince, and the mixture was stirred with a magnetic flea for 1 hour at room temperature. The flask was then slanted to permit the sedimentation of larger tissue particles and the supernatant fluid was decanted through a 72-mesh stainless steel wire cloth in a 250-ml centrifuge bottle. The cell suspension was *immediately* centrifuged horizontally at 600 to 700 rpm for 10 minutes, after which the supernatant fluid was removed and replaced with an equal volume of Hanks' BSS. The cells were resuspended in this rinse fluid and then sedimented by additional centrifugation at 600 to 700 rpm for 10 minutes. After the rinse fluid was aspirated, the packed cells were resuspended in 15 to 30 ml of Hanks' BSS and transferred to 15-ml conical, graduated centrifuge tubes. The cells were packed by centrifugation at 600 rpm for 10 minutes, and the volumes of packed cells were noted. After removal of the supernatant fluids, a 1:200 dilution of the cells was prepared in the outgrowth medium described below.

Before these comparative studies were initiated, preliminary investigations were conducted, aimed at devising a procedure for the preparation of chick embryo cell cultures which would be relatively simple (*i.e.*, would not involve multiple feedings of the cell cultures or extensive washing of the cultures before addition of maintenance medium and inoculation) and which would produce a monolayer of cellular growth that would hold up during 7 or more days of observation after inoculation with the test materials.

Of 17 different outgrowth media which were compared, the medium found to be most satis-

§ Volumes of 0.25% trypsin solution used for dispersal of chick embryo cells were 200 ml for 5 to 10 embryos, 300 ml for 11 to 20, and 400 ml for 21 to 30.

factory, both from the standpoint of initiating cellular growth and conditioning the cell sheets to hold up during the observation period, was Hanks' BSS containing 2% lamb serum, 0.25% lactalbumin hydrolysate, and 2% chick embryo extract. This medium was buffered by the addition of 2.5 ml of a 2.8% solution of $NaHCO_3$ per 100 ml, and antibiotics were added to give a final concentration of 250 units of penicillin, 250 μg of streptomycin, 250 units of neomycin and 2.5 units of bacitracin per ml.

The inclusion of chick embryo extract in the outgrowth medium was found to be important in conditioning the cells to hold up during the 7-day observation period after the addition of maintenance medium and inoculation.

The lamb serum was previously found to be free from antibodies or inhibitory substances for WEE, SLE, and Turlock viruses, and, therefore, it was not necessary to wash the cell sheets before addition of maintenance medium and inoculation with virus.

Tubes were seeded with 1.0 ml of the 1:200 dilution of cells in the outgrowth medium and incubated at 36°C. After 1 to 2 days of incubation, the tubes contained confluent sheets of cells, and the outgrowth medium was decanted and replaced with 1.0 ml of maintenance medium.

Seven different media were compared for their ability to maintain cultures of chick embryo cells for the required observation period, and the most satisfactory was found to be Eagle's basal medium (prepared in Hanks' BSS) buffered by the addition of 3.0 ml of a 2.8% solution of $NaHCO_3$ per 100 ml of medium. Antibiotics were added to the maintenance medium in the same concentrations as those used in the outgrowth medium.

Preparation of hamster kidney tissue culture. The method employed for the preparation of tube cultures of hamster kidney cells was essentially that described by Kissling,[1] with the exceptions that the outgrowth medium consisted of lactalbumin hydrolysate-yeast extract medium‖ with a 10% concentration of calf serum, and the maintenance medium consisted of lactalbumin hydrolysate-yeast extract medium with a 3% concentration of calf serum (inactivated at 56°C for 30 minutes). The maintenance medium was further buffered by the addition of 1.0 ml of 8.8% $NaHCO_3$ per 100 ml. Both media contained 250 units of penicillin and 250 μg of streptomycin per ml.

The hamster kidney cell cultures were initiated by seeding tubes with a 1:125 dilution of packed cells in a volume of 0.8 ml of outgrowth medium. After the cultures had incubated at 36°C for 2 days, an additional 1.0 ml of outgrowth medium was added to each tube and incubation was continued. After a total of 5 days of incubation the outgrowth medium was removed, 1.0 ml of maintenance medium was added, and the cultures were inoculated with mosquito pool suspensions or with passage material.

‖ The lactalbumin hydrolysate-yeast extract medium has the following composition: lactalbumin hydrolysate, 5.0 g; glucose, 4.5 g; yeast extract, 1.0 g; $NaHCO_3$, 1.1 g; phenol red, 1.0% aqueous solution, 1.6 ml; and Earle's balanced salt solution (without $NaHCO_3$) to 1,000 ml.

RESULTS

Toxicity of mosquito pool suspensions for the four host systems. Mosquito pool suspensions prepared as described in the section on Materials and Methods possessed no obvious toxicity for embryonated eggs and mice; in examination of 295 mosquito pools in this study, none was found toxic for these host systems. On the other hand, certain pools proved toxic for the two tissue culture systems; the toxic factor, however, was in all instances diluted out by the second passage. In comparing the sensitivity of chick embryo and hamster kidney tissue cultures to the toxic effects of mosquito pool suspensions at the first passage level, only those mosquito pools which failed to yield a viral agent in any of the host systems are considered, as it is usually not possible to distinguish between toxicity and specific cytopathogenic activity in instances where a viral agent is recovered. Of 252 mosquito pools from weekly collections made in Sutter County during the 1958 study year, 215 failed to yield a viral agent in any of the four host systems. That chick embryo tissue cultures were considerably more susceptible to the toxic effects of the mosquito pool inocula than were hamster kidney tissue cultures, is indicated by the fact that 100, or approximately 47%, of the pools were toxic at first passage in chick embryo cells, while only 20, or approximately 9%, of the pools were toxic for hamster kidney cells.

It was considered desirable that in those instances in which an inoculum was toxic for a tissue culture system, causing cellular degeneration and apparently cellular death within the

first 48 hours, the material should be passaged into fresh cultures as soon as possible in order to prevent viral agents which might be present from dying due to lack of viable host cells. Consequently, cultures which manifested degeneration (either specific or toxic) within 48 hours after inoculation, were immediately passaged into cultures of the same cell type. This procedure also speeded the identification of certain of the isolates recovered in tissue cultures, as will be discussed below.

Sensitivity of the four host systems for the isolation of viruses. In addition to the 252 mosquito pools from the weekly collections made in Sutter County, 43 other pools were examined in the comparative studies, making a total of 295 pools which were inoculated in parallel into the four host systems. These additional 43 pools contained, in addition to *Culex tarsalis*, a number of *C. stigmatosoma* and *C. pipiens*, and were collected in other counties in the San Joaquin Valley. Table 1 compares the number of viral agents isolated in each host system from these 295 pools.

For the isolation of WEE virus, embryonated eggs were somewhat more sensitive than the other three host systems, as 27 isolates were recovered in eggs, as contrasted to 24 in mice, and 22 in each of the tissue culture systems. The same five isolations were missed in both tissue culture systems, and two of these pools were also negative in mice. Isolates recovered in eggs from three of these five pools could not be identified as WEE virus until a second egg passage was made. It would thus appear that these five pools contained minimal amounts of virus, and that the virus was detected only in the most sensitive host system.

As is shown in Table 1, more strains of SLE virus were recovered in mice than in other host systems. The same four mosquito pools yielded SLE virus in eggs and chick embryo tissue cultures; however, these agents did not establish themselves in these hosts, and it was necessary to pass them to mice so that they could be identified in neutralization tests in mice. Five of the seven mosquito pools which yielded SLE virus in mice also yielded this agent in hamster kidney tissue cultures. These isolates were identified in neutralization tests in mice and in monolayer cultures of hamster kidney cells.

Although chick embryo tissue cultures yielded only one less SLE virus isolate from the 1958 pools than did hamster kidney cells, the virus, even after three passages in this tissue, did not produce marked cytopathic effects and could be identified only after passage to mice. Table 2 compares the reactions of the five isolates in both tissue culture systems at the three passage levels, and it is seen that they failed to produce three plus or four plus cellular degeneration in chick embryo cells, whereas in hamster kidney complete degeneration was produced after 2 to 3 passages. It should be pointed out, however, that in no instance did an SLE isolate produce marked cellular degeneration in hamster kidney cells at the first passage level, and the isolates were recovered only after further passage in this tissue.

TABLE 1

A comparison of the sensitivity of four host systems for the isolation of Western equine encephalomyelitis, St. Louis encephalitis and Turlock viruses from mosquito pool suspensions

Virus isolations	Host systems			
	Embryonated eggs	Mice	Chick embryo tissue cultures	Hamster kidney tissue cultures
No. pools tested	295	295	295	295
No. pools positive for WEE virus	27	24	22	22
No. pools positive for SLE virus	4	7	4	5
No. pools positive for Turlock virus	8	0	2	0

TABLE 2

Recovery of St. Louis encephalitis virus from 1958 mosquito pools in chick embryo and hamster kidney tissue cultures

Mosquito pool number	Degree of cellular degeneration after 5–7 days' incubation in					
	Chick embryo tissue cultures Passage number			Hamster kidney tissue cultures Passage number		
	1	2	3	1	2	3
50–8	toxic	±	2+	±	4+	
56–2	2+	2+	1+	0	1+	3+
56–12	toxic	±	2+	±	2+	3+
58–2	±	2+	2+	±	1+	4+
61–12	toxic	±	±	±	4+	

In Table 1 it is seen that the only suitable host for the isolation of Turlock virus was the embryonated egg, as neither mice nor hamster kidney tissue cultures yielded a Turlock virus, and only two of the eight pools which yielded a Turlock virus in embryonated eggs were also positive in chick embryo tissue cultures. However, it was found that chick embryo tissue cultures could be used to advantage in the identification of Turlock viruses by the complement fixation technique. This will be discussed in the following section.

The number of SLE and Turlock virus isolates recovered from mosquito pools during the 1958 study year was small, and in order to compare further the sensitivity of the four host systems to these viruses, pools from the 1957 study year previously found to contain the SLE and Turlock viruses were reinoculated into the four host systems. Results of these reisolation attempts made 1 year later are presented in Table 3.

It was found that both SLE and Turlock viruses in the mosquito pools were extremely labile, even when stored at −70°C. Only 17, or approximately 59%, of the pools positive for SLE virus in 1957 yielded the virus on retesting a year later, and only 7 of the 37 pools positive for Turlock virus in 1957 yielded this virus in the later isolation attempts.

Table 3 shows that hamster kidney tissue cultures yielded one isolate more from these 1957 mosquito pools than did mice. Two isolations of SLE virus were made in mice which were not made in hamster kidney tissue cultures, and three isolates were recovered in hamster kidney cells which were not recovered in mice. Embryonated eggs yielded three isolates which were not recovered in the mouse or in hamster kidney tissue culture systems. No isolations of SLE virus were made in chick embryo tissue cultures.

On the basis of the data obtained from tests on mosquito pools from the 1957 and the 1958 seasons, hamster kidney tissue cultures were comparable in sensitivity to mice for the isolation of SLE virus. However, it should be emphasized that none of the isolates of this virus were cytopathogenic in hamster kidney tissue cultures at the first passage level and were recovered only by making successive blind passages.

As was the case with the 1958, *i.e.*, freshly prepared mosquito pools, embryonated eggs were the only hosts that proved adequate for the recovery of Turlock virus from the 1957, *i.e.*, stored mosquito pools. None of the seven 1957 pools positive for Turlock virus in embryonated eggs yielded the virus in any of the other three host systems.

TABLE 3

A comparison of the sensitivity of 4 host systems for the reisolation of St. Louis encephalitis and Turlock viruses from mosquito pools

Number pools positive for SLE virus on		Number pools positive for SLE virus on retest in			
Original test	Retest 1 yr later	Embryonated eggs	Mice	Chick embryo tissue cultures	Hamster kidney tissue cultures
29	17	6	11	0	12
Number pools positive for Turlock virus on		**Number pools positive for Turlock virus on retest in**			
Original test	Retest 1 yr later	Embryonated eggs	Mice	Chick embryo tissue cultures	Hamster kidney tissue cultures
37	7	7	0	0	0

Identification of viruses in the different host systems. In this laboratory, WEE viruses isolated in embryonated eggs are routinely identified by a complement fixation technique employing the infected membranes and fluids as an antigen.[7] By this method, identification can usually be accomplished with first passage material within a week after recovery of the agent. In the course of these studies, it was found that fluids from both chick embryo tissue cultures and hamster kidney cultures infected with WEE virus could be employed as complement-fixing antigens for identification purposes. In order to speed identification of WEE isolates recovered in tissue cultures (and to circumvent toxicity of the inocula) pools producing three plus or four plus degeneration 48 hours after inoculation were immediately passaged into eight tissue cultures of the same cell type (and of the same lot as those initially inoculated, but held on maintenance medium in the interim). If the second passage material caused three plus or four plus degeneration after 72 hours, the fluids were employed as complement-fixing antigens in tests against WEE immune sera. Thus, identification of WEE isolates from tissue cultures could also be accomplished within 1 week if the isolates at first

passage caused sufficient cytopathogenic degeneration during the first 48 hours to warrant making a second passage for preparation of a complement-fixing antigen.

It was found that WEE virus isolates caused cytopathic changes earlier in chick embryo cells than in hamster kidney cells, and for this reason an early identification could be accomplished more frequently from infected chick embryo tissue culture fluids than from hamster kidney fluids. This is illustrated in Table 4, where it will be noted that 20 of the 22 isolates recovered in chick embryo tissue cultures were identified within the first week, while only 12 of the 22 isolates recovered in hamster kidney cells caused cytopathogenesis early enough to be passaged after the first 48 hours, and thus to be tested within the first week.

Neutralization tests in mice for the identification of isolates of SLE virus were performed on second and third mouse passage material; the test animals were observed for a total of 14 days. The identification of SLE virus by neutralization tests conducted in hamster kidney cells was somewhat more rapid than in neutralization tests done in mice. Although two or more passages in hamster kidney cultures were required before the isolates possessed sufficient cytopathic activity to be used in neutralization tests, the tube neutralization test itself gave more rapid results since readings were made over only a 7-day period.

The identification of isolates of Turlock virus has posed problems in that infected egg materials do not yield complement-fixing antigens satisfactory for identification by this technique,[8] and hence identification of this agent has depended upon neutralization tests done in infant mice. However, it was found in the course of these studies that fluids from chick embryo tissue cultures infected with Turlock virus possessed a low level of specific complement-fixing activity, and that the agent could be identified by testing the infected fluids for specific complement-fixing activity with hamster immune serum. The Turlock viruses isolated in chick embryo tissue culture were identified by this method, and the isolates recovered in embryonated eggs were passed to chick embryo tissue cultures and identified by complement fixation tests on the infected culture fluids. This was less cumbersome and time-consuming than neutralization tests in mice.

TABLE 4

Rapidity of identification, by complement fixation test, of Western equine encephalomyelitis viruses recovered in three different host systems

Weeks from isolation to identification	No. virus strains recovered in		
	Embryonated eggs	Chick embryo tissue cultures	Hamster kidney tissue cultures
1	23	20	12
2	4	2	7
3			1
4			2
Total isolated	27	22	22

DISCUSSION

The two tissue culture systems studied were satisfactory for the isolation of WEE and SLE viruses from mosquito pool suspensions, but they were somewhat less sensitive than embryonated eggs for the isolation of WEE virus. It would appear that hamster kidney tissue cultures would be more valuable than chick embryo tissue cultures for isolation of these agents, as both the WEE and SLE viruses were clearly cytopathogenic for this cell type. The SLE virus did not produce complete cellular degeneration in chick embryo fibroblasts even after three passages. The isolates of WEE virus recovered in this study did not produce cytopathogenesis as rapidly in hamster kidney as in chick embryo tissue cultures, but hamster kidney cultures permitted the recovery of the same number of WEE virus isolates as did chick embryo cell cultures, and the WEE virus strains recovered in both cell types could be rapidly identified by a complement fixation technique. The SLE viruses recovered in hamster kidney tissue cultures could be identified by a neutralization test in monolayer cell cultures, which was less cumbersome and gave a more rapid answer than did neutralization tests in mice. Another advantage in the use of hamster kidney tissue cultures over the use of chick embryo cell cultures was the fact that the former cell type was less susceptible to the toxic effect of the mosquito pool inocula.

Neither tissue culture system was found to be suitable for the isolation of the Turlock virus, as no isolations of this agent were made in hamster kidney cells and only two of the eight 1958

isoltaes recovered in eggs were also recovered in chick embryo tissue cultures. None of the Turlock viruses from the 1957 mosquito pools were recovered in either tissue culture system. However, it was found that this agent could be identified from a complement-fixing antigen derived from chick embryo tissue cultures. This was particularly interesting in light of the fact that it has been impossible to demonstrate satisfactory complement-fixing antigens for this virus in the fluids or membranes of infected eggs.[8]

Although tissue culture techniques possess certain advantages[9] over the use of animals and eggs for virus isolation purposes inasmuch as they eliminate the need for large animal quarters, the feeding and care of animals, candling of eggs, and the grinding of infected egg membranes and animal tissues, their use cannot be considered inexpensive since the preparation of glassware and media, the initiation and feeding of cell cultures, and the microscopic observation of inoculated cultures all require considerable personnel time. Further, the occurrence of non-specific degeneration in cell cultures (caused either by toxic inocula or aging of the cell cultures) frequently leads to difficulty in interpretation of results; this difficulty was encountered to a variable extent with the method described in another report[9] from this laboratory.

It appears from these studies that if tissue culture systems are employed for the isolation of viral agents from mosquito pools, it is necessary to passage the test materials two to three times, not only because of the toxic effect of many of the inocula at the first passage, but also because some of the agents recovered in tissue cultures gave no evidence of their presence at the first passage level. Thus, none of the five isolates of SLE virus recovered in hamster kidney from the 1958 study material caused more than ± degeneration after 7 days of incubation at the first passage level, and only two of these isolates produced adequate cytopathic changes at the second passage level to permit their identification. None of the SLE isolates from the 1957 mosquito pools could be identified before the second or third passage in hamster kidney tissue cultures.

The fact that test materials in tissue culture systems must be carried through three passages before they may be considered to be negative may actually make these host systems more cumbersome to employ than mouse and egg systems, as repeated passage of negative materials is involved, whereas with the use of the mouse and the embryonated egg, test materials which do not display pathogenic activity at the first passage level are considered negative. In these studies on the 1958 mosquito pools, blind passage of materials in the two tissue culture systems did not provide a more sensitive detection method than that available with mouse and egg systems, as no isolates were recovered in tissue culture systems which were not also recovered in either embryonated eggs or mice.

Three of the 1957 mosquito pools yielded an SLE virus in hamster kidney tissue culture, but not in mice. However, in view of the lability of the virus under the conditions of storage, it is uncertain whether the recovery of these particular isolates was due to a greater number of passages in the culture system or whether such small amounts of virus were present in these pools that each host system received different concentrations of virus in the inoculum. The mouse and embryonated egg systems both yielded isolates which were not recovered in the other host systems.

It would appear that mice and eggs are the host systems of choice for the isolation of arthropod-borne viruses, because of the somewhat greater sensitivity of these systems and the fact that negative materials need not be passaged more than once. Tissue cultures systems are useful to expedite identification of the agents; *e.g.*, complement-fixing antigens derived from chick embryo tissue cultures may be used for the identification of Turlock virus or the SLE virus can be identified by neutralization tests in hamster kidney tissue cultures. Where facilities for egg and mouse work are unavailable or not adequate, recourse may be had to hamster kidney tissue culture procedures, which appear to be adequate for the recovery of SLE and WEE virus isolates from field materials.

SUMMARY

Mosquito pool suspensions were inoculated in parallel into embryonated hens' eggs, mice, chick embryo tissue cultures and hamster kidney tissue cultures for a comparison of the sensitivity of these four host systems for the isolation of St. Louis encephalitis, Western equine encephalomyelitis and Turlock viruses. Mice and hamster kidney tissue cultures were the most sensitive

hosts for the recovery of SLE virus, and approximately the same number of isolates were recovered in each of these systems. Embryonated eggs were slightly more sensitive than the other three host systems for the isolation of WEE virus. The only satisfactory host system for the isolation of Turlock virus from the mosquito pools tested was the embryonated egg.

The facility of identification of the isolates in the different host systems is discussed.

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EFFECTIVENESS OF TRAPS IN TSETSE SURVEYS IN THE LIBERIAN RAIN FOREST*

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In 1959 The American Foundation for Tropical Medicine started an investigation of the sleeping sickness problem in Liberia, under the auspices of its Liberian Institute and operating from a Field Station at Voinjama in the northwestern corner of this country, where the disease had been epidemic in 1940–44.[1] An essential part of the undertakng was to make a survey of the tsetse fly vectors of *Trypanosoma gambiense*, the causal agent. Since the eventual aim was to draw up recommendations for control, which must be based on a knowledge of epidemiology, the survey was planned to give something more than the distribution of tsetse fly on a mere presence or absence basis. It was necessary to know the relative abundance of the vectors, particularly in the affected or suspect areas, and if possible to give this in terms of man-fly contact, the vital factor in the epidemiology of human trypanosomiasis. It was also necessary to be able to make observations over wide areas of the country and at different seasons of the year which would be strictly comparable, and for this a method of survey which was simple yet standard in technique was essential. The usual method of survey, by hand-catching with trained teams of local Africans, or "fly-collectors," was difficult to apply; the men needed long periods of training and much weeding out for reliability, largely because of the low standard of education which prevailed. Consequently it was decided to attempt both the wider survey and the detailed local investigations by means of traps, which would fulfill the need for standardization and would overcome the difficulty of obtaining reliable observers, since one good observer could take charge of a large number of traps. Furthermore the writer's experience elsewhere[2] had shown that traps could be more efficient in sampling the populations of a number of species of *Glossina* than even the most skilled fly-collectors.

The whole of Liberia lies within the Guinea Zone of tropical rain forest, although in the north, where most of the sleeping sickness had occurred, shifting cultivation by a fairly abundant population had left little of the original climax vegetation. This was replaced by secondary forest, showing all stages of recovery after farming, from high forest with almost completely closed canopy to open pseudosavanna, covered by low bush and herbaceous growth with high forest confined to narrow belts along the rivers and streams. This riverine gallery forest frequently constituted the habitat of *Glossina palpalis* R.D., the known vector of *T. gambiense*.

Work in the Gold Coast[3] had shown that a simple form of unbaited trap, Morris's animal trap, was effective in catching *G. palpalis* in dry open savanna woodland country and in the true rain forest of Ashanti. The conditions there were very different from those of Liberia, both vegetationally and climatically. Therefore, experimental work was required on three lines: the performance of the trap, or of modifications, under Liberian conditions, its suitability for measuring the factor of man-fly contact, and the most suitable techniques for its use in sampling and in survey. Since the writer was in Liberia for only 12 months, it was necessary to limit the work to practical issues which would contribute directly to the formulation of a reliable method of survey.

The Type of Trap

The form of trap which was most successful in catching *G. palpalis*, *G. tachinoides* Westw, *G. longipalpis* and the *fusca* group of tsetse in the Gold Coast has been described and illustrated elsewhere.[3] The target which attracts the flies is a cylindrical body, 2 feet long by 1 foot in diameter, standing with the top or shoulder 2 feet from the ground, and covered with ordinary light brown hessian or burlap (Figure 1). Al-

* The studies reported in this paper were financed by the International Cooperation Administration, an Agency of the United States Government, but do not necessarily represent the decisions or the stated policy of the International Cooperation Administration.

FIG. 1. The "Morris" tsetse trap in a good site at the water's edge in an open feeding ground of *Glossina palpalis*, with the evergreen fringing forest of the fly's habitat beyond. The trapping site is marked with a numbered peg, and the trap anchored with a rope in case of a sudden rise in river level.

though the traps were originally designed to imitate a natural food host and covered with the skins of antelope, goats or sheep, it was found that hessian was equally effective and was cheaper and more durable in practice. Two modifications were tried in Liberia, those of color and of size.

Black traps appeared more obvious to the human eye, but in a 10-weeks' trial in which black and natural hessian covered traps were rotated around the same sites in a good *G. palpalis* fly belt 30 miles south of Voinjama, no difference was found: one natural hessian trap caught 129 flies, with 60% females and one black hessian trap caught 132 flies, with 61% females. It was observed, however, that certain species of tabanids showed a preference for dark over light colored traps.

Since observations showed that in the unpopulated forest of Liberia the pigmy hippopotamus (*Chaeropsis liberiensis*) and the dwarf buffalo (*Syncerus nanus*) were probably fed upon by *G. palpalis*, experiments were tried with traps double the standard size, *i.e.*, 4 feet long and 4 feet high at the shoulder, which approximated more closely the size of these hosts. Trials were made on the Lawa River, in an almost unpopulated high forest south of Voinjama, first in November, 1959, at the end of the wet season, and repeated in January through March, 1960, during the dry season. On both occasions the large traps made higher catches than small traps. The difference was greater in the dry season, as the following figures show: In November and December, 1959, at the end of the rains, one small trap in 6 weeks caught 365 flies (67% females), or 60.8 flies per trap per week, and one large trap in 5 weeks caught 343 flies (60% females), or 68.6 flies per trap per week, giving a superiority of 13%. From January through March, 1960, the dry season, two small traps in 10 weeks caught 261 flies (61% females), or 13 flies per trap per week and two large traps in 10 weeks caught 400 flies (53% females), or 20 flies per trap per week, giving a superiority of 54%. For some reason which is not understood the large traps were less attractive than small traps to female flies. Their difference in this respect was constant during both seasons despite the variation in their comparative rates of catching.

In the second trial one large trap was covered with natural hessian and one with black, but their performances were absolutely equal.

It was found that there was such a variation in the performances of individual traps when made in the double size that standardization was difficult to maintain. They are also more costly in material and difficult to carry about. Consequently, in operations where numbers of traps are in use and strict comparability is essential, these large traps are not recommended, despite their better catching power. For special work, when large numbers of flies are required, they would be useful. They also proved more attractive than small traps to certain species of Tabanidae and for use in the study of this family they would certainly be valuable.

Variation in the performance of individual traps was found also in the small size, particularly among traps made by different workmen. To maintain a standard of accuracy in this work, it is necessary to have trap making under the charge of one man who should have field experience with traps, and be fully aware of the small details which contribute to their efficient performance.

A Portable Folding Trap

The disadvantage of the standard form of trap as an instrument in survey was its bulkiness, which made it difficult to transport in the numbers needed for effective survey purposes. To overcome this drawback a folding trap was

designed. Details are shown in Figures 2, 3, and 4.

The catching cage remains unaltered, a rectangular cage 18 inches long with wooden frame covered with wire gauze and with a sleeved opening at one end to allow removal of the catch. Sockets at the ends allow it to be mounted on two pairs of legs made from one-fourth inch reinforcing steel held together by welded cross pieces. The cloth covering, shaped as in the sketch, is kept open by five wire rings 12 inches in diameter, sewn into the cylindrical bag. This can be drawn over the legs and tied in place with draw tapes around the base of the cage. When removed, it folds flat like a concertina. Three or four of these folding models are as easily carried as one standard trap.

In operation, the type of material covering the body is all important. At first, a light brown drill was used for ease of sewing and for lightness when packed. These folding traps caught *G. palpalis*, but their catches were only 20% of the catches made by standard traps working at the same place and time. As a rough surface material is known to be more attractive to tsetse, traps covered with dark brown velvet were next tried and gave better results, but still well below those with standard traps. Finally folding traps were covered with ordinary hessian, and in a short series of trials these caught almost as well as the standard model. The experiments left no doubt that a folding trap of this or similar type, hessian covered, would be extremely useful in tsetse survey.

The Measurement of Man-Fly Contact

A simple measure of the factor "man-fly contact" could be given by fly-collectors posted at points near a village where people come in contact with *G. palpalis*, such as fords, bridges, water holes and washing places, and catching all tsetse coming to attack the people or themselves. The possibility of using traps for such measurements would depend on whether their catches under such circumstances were equal to those made by fly-collectors. The first experiment to test the performance of traps in this respect was made at the main point of contact of the people of Voinjama with *G. palpalis*, on the Zeliba River where it is crossed by a main road bridge 1.5 miles south of the town. The dense riverine habitat of *G. palpalis* was broken here by an open clearing 70 yards long, which, with the constant presence of people crossing the river, washing clothes, or bathing, constituted an ideal feeding ground for the tsetse (Figure 5). Eight traps were placed here at the beginning of June 1959, four in the feeding ground and four in fly-belt and were cleared twice a day (7 a.m. and 1 p.m.) by a supervisor. Two collectors were

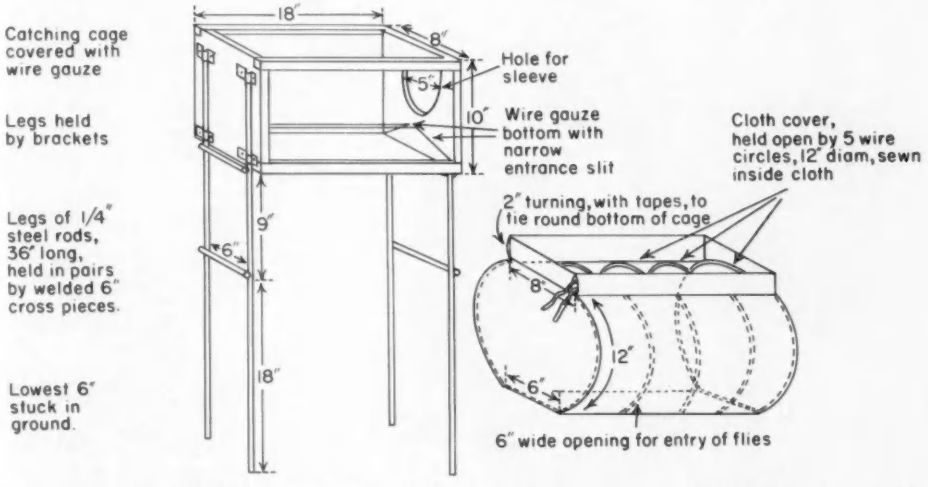
Catching cage covered with wire gauze
Legs held by brackets
Legs of 1/4" steel rods, 36" long, held in pairs by welded 6" cross pieces.
Lowest 6" stuck in ground.
18"
8"
Hole for sleeve
5"
10"
Wire gauze bottom with narrow entrance slit
9"
6"
18"
Cloth cover, held open by 5 wire circles, 12" diam, sewn inside cloth
2" turning, with tapes, to tie round bottom of cage
8"
12"
6"
6" wide opening for entry of flies

FIG. 2. Details of the folding trap. The catching cage is shown mounted on the two pairs of removable legs. The cloth body, which folds concertina-wise, is shown separately.

stationed here under the same man in July. They searched the area sampled by the traps from 7 a.m. to 1 p.m. 6 days a week. Work continued until the writer left Voinjama in May, 1960, so that 10 months of uninterrupted man and trap catches were available for comparison.

FIG. 3. The folding trap in operation

The total catches of *G. palpalis* for the period covering 42 weeks were as follows: eight traps caught 82 males, 268 females, a total of 350 flies (76.6% females), and two men caught 88 males and 166 females, a total of 254 flies (65.4% females). Thus, traps caught 138 flies for 100 caught by men. This one-third superiority of traps over men is made up entirely by the greater number of female *G. palpalis* caught by traps, a phenomenon which occurred also in the Gold Coast[3] and in Uganda with *G. pallidipes*.[4] A possible explanation lies in the fact that female tsetse have much briefer periods of activity than do males, appearing only when hungry and in search of food, whereas, the males range in search of mates as well as for food. Traps, then, which are continually present in the fly-belt, in contrast to the much shorter visits by collectors, have the greater opportunity of catching the more elusive female flies when they do appear.

More information can be obtained by breaking

FIG. 4. The folding trap dismantled, also one erected beside a standard model.

down these figures into monthly totals, as shown in Table 1 and Figure 6, in which the catches are standardized to 32 trap-weeks and 8 man-weeks per month. Table 1 brings out an important point in trapping which was observed in the dry savanna country in the north of the Gold Coast,[3] that the higher catching power of traps compared with collectors was most marked in the dry season and disappeared during the rains. Two factors work to the advantage of traps in the dry season: The concentration of the *G. palpalis* populations in their narrow riparian habitat, bringing them into close contact with traps well sited along the river banks, and the very variable activity periods of this fly, which may be restricted to a few hours a day in the dry season.

FIG. 5. Voinjama town washing place, in an open space in the fringing forest habitat of *Glossina palpalis* on the Zeliba River. In this feeding ground of *G. palpalis* a 10-month's comparison between traps and fly-boys was made.

TABLE 1

Monthly catches of Glossina palpalis *by traps and by fly-collectors on the Zelib River near Voinjama, Liberia, July 1959 to April 1960*

	8 traps	2 men	Traps as % of men	Conditions
July	32	44	72.7	Rain
Aug.	24	35	68.6	Heavy rain
Sept.	17	20	85	Rain
Oct.	49	28	175	End of rains
Nov.	42	29	145	Dry
Dec.	52	32	172	Dry
Jan.	23	15	153	Dry
Feb.	20	18	112	Dry
Mar.	28	5	560	Few storms
Apr.	37	11	336	Few storms

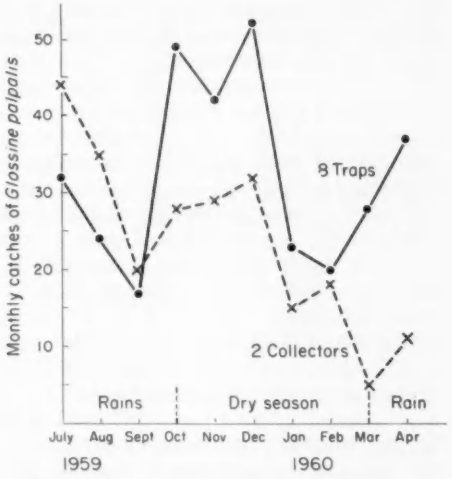

FIG. 6. The comparison of traps with human observers for sampling *Glossina palpalis*, made by simultaneous catching in the tsetse's feeding ground on the Zeliba River near Voinjama, Liberia, July 1959 to April 1960.

Traps, in contact with the fly 24 hours a day and 7 days a week, will benefit by this activity whenever it occurs, whereas the collectors, with their limited hours of catching, may miss it completely. The advantage of concentration is lost in the rains, when the more abundant vegetation cover and the less extreme climatic conditions result in a much wider dispersal of *G. palpalis* which will now occupy many secondary habitats well away from the river, difficult to sample by fixed traps but more easily located and searched by mobile collectors.

In this Voinjama experiment four-fifths (277) of the trapped flies were taken by the four traps sited in the open *G. palpalis* feeding ground close to the bridge. Thus, it was shown that under the conditions of low tsetse populations on a small river, four well sited traps could equal the catch made by two collectors, and that during the dry season these traps demonstrated the presence of considerably more flies than could be detected by men.

Similar comparisons were made at two other places, one where *G. palpalis* was present at very low density, and another where this fly was more abundant.

At the village of Vezala, on the same river 12 miles east of Voinjama, 12 weeks catching in the

dry season (Jan.–Mar., 1960) by four traps and two men, working simultaneously at village water holes and river crossings, gave the following numbers of *G. palpalis:* four traps caught 38 males and 71 females, a total of 109 flies, or 65.1% females, and two fly-collectors caught 25 males and 40 females, a total of 65 flies, or 61.6% females, which gives a ratio of 152 caught by traps for 100 caught by men. The ratio here is close to that shown at the same time of year at Voinjama. Again traps took more female flies, though the disparity is not so great as in the former experiment.

The second example comes from the Lawa River, which held a good community of *G. palpalis* at the time of the experiment, early in the dry season at the end of 1959. For 4 weeks of November and December, 1959, two fly-collectors worked here 6 days a week, and their catches can be compared with those of two standard traps from a series then being tested at this place as follows: two traps caught 123 males and 289 females a total of 412 flies, or 70.1% females, and two men caught 97 males and 137 females, a total of 234 flies or 54.3% females. Thus, two traps caught 176 flies for 100 caught by two men. This remarkably good performance for traps compared with men, double the superiority shown at Voinjama for the same period, may be explained by the much larger numbers of flies present. It takes a fly-collector a little time catching a tsetse, and then transferring it from net to box; tsetse are quick and elusive insects. Two or three flies may enter a trap at the same time, and have been seen to do so. In other words, there is a limit to the speed of catching with men and no limit in the case of traps. Thus, the superiority of traps to men shown in sampling tsetse at low concentration becomes greater as higher densities of fly are encountered.

There is one other set of conditions under which traps have a great advantage over men. The dispersal of *G. palpalis* away from the main rivers during the rains might bring flies into dangerously close contact with villages, a point of importance epidemiologically. North of Voinjama, and only 800 yards from the town, was a much frequented water hole and washing place in a marsh where permanent habitat for tsetse was absent, but where occurrence of *G. palpalis* was suspected. Two traps were kept by the water hole for 11 months, from June 1959 to May 1960, during which time nine *G. palpalis* were captured. These were all caught during the first 4 months, June to September, at the height of the rains, and not a single fly was taken in the next 7 months. Fly-collectors worked here for a week each month in July, August, and September without seeing a tsetse. Doubtless they would have caught some had they been stationed continuously at this place, but that would have been a great waste of personnel. Moreover, the very rare appearances of fly, with 13 days before the first was caught and a further 26 days to the second, would inevitably result in loss of interest and skill in the team. Visiting these two traps took up 10 minutes daily of the time of a trap man who was operating twelve more traps in other places at the same time. Incidentally, a long series of Tabanids, including a species of *Chrysops*, was captured in these two traps.

Although time did not allow the full series of experiments necessary to establish an exact relationship between trap and human catches, at least it was shown that groups of two to four traps, well-sited at points of man-fly contact, made catches equal to, and in some cases greater than those made by a team of two collectors. Moreover, traps would give a measure with a higher standard of reliability over the whole range of frequency at which *G. palpalis* occurs, from the very occasional fly to the abundant community in which the man's catching power would be strained. From the practical angle, traps would be far more economical than collectors. One trap-man would be able to operate without difficulty twenty traps and sample, say, five different localities simultaneously.

Frequency of Visits

Since this type of trap catches continuously, without any manipulation beyond that necessary to keep it in good repair, it would be possible for two or three times the number of traps to be operated by a single man if his visits were at 2- or 3-day intervals instead of daily. However, it was known that some tsetse escape again after entering these traps, so it was important to find out what the effect of different frequencies of visits would be on the relative size of catches.

The first approach was to analyze the catch made over 5 months at two places in which traps

were visited daily with the exception of Sundays, and compare Monday catches, which represented a 2-day interval between visits, with daily catches from Tuesday to Saturday. In daily visits, 230 trap-days gave 203 *G. palpalis*, or 0.88 flies per trap per day, and in 2-day visits, 93 trap-days gave 55 *G. palpalis*, or 0.59 flies per trap per day. Thus, 2-day visits yielded only two-thirds as many flies as daily visits. The percentage of female flies, 74.2% in daily visits and 69% in 2-day visits, was not significantly different for the two methods.

An experiment was then undertaken in a good *G. palpalis* fly-belt on the Lofa River 18 miles south of Voinjama, in which six traps were arranged in three groups. Group 1 was visited twice daily, group 2 daily, and group 3 every 2nd day. The routine was rotated at weekly intervals around the three groups. Thus, every 3 weeks each group had each type of treatment for a 5-day period. In the analysis, Monday catches from all traps were grouped with the data from 2-day visits. The experiment lasted 12 weeks, during which time traps were rotated around the sites to cancel out any individual trap variations. The results, shown in Table 2, clearly indicate that visits on alternate days gave a figure close to that arrived at in the first analysis, roughly 70% of the catch recorded with daily visits.

The twice daily visits show the operation of another factor besides that of escape. It is well known that the presence of the tsetse's hosts close to traps brings an increase in their catches.[5] In the present experiment the first visit to all traps was between 7 and 8 a.m. when relatively few active flies would be seen; the second visit to the twice-a-day group was at midday, when *G. palpalis* was active, and hungry flies would be attracted to the traps by the presence of the supervisor, often accompanied by his fly-collectors or by one or two curious passersby. This occurrence was repeatedly observed by the men and by the writer, and it was further observed that the non-hungry male flies often departed with the men after the trap was cleared, whereas the hungry females tended to linger around the traps and eventually get caught. This tendency would explain the high percentage of females caught on twice daily visits. In these samples the proportion of female flies taken is probably higher than the true index for the natural tsetse population, which will be nearer to that shown in samples taken by daily visits, *i.e.*, between 60 and 70% females.

A second experiment was carried out to test the efficiency of trapping routines with three visits or two visits per week against standard daily visits. Twelve traps were arranged in three groups. All traps were visited daily except on Sundays, but in group 1 flies were removed on each visit; whereas, in groups 2 and 3 the traps were cleared three times and twice a week respectively, and on the other days the number of flies seen in the traps was noted down but they were not removed. The three different procedures were rotated around the groups of traps weekly, and traps were rotated around sites at weekly intervals. The experiment was undertaken on the Lawa River, for 8 weeks, March to May 1960. Total catches of *G. palpalis* for the period (number of trap-days equal for each group) were as shown in Table 3. Since the effect of the presence of men was uniform, with all traps visited daily, the difference in catches between the groups must be due to escape only.

TABLE 2

Comparison of catches of Glossina palpalis *taken from traps visited twice daily, daily, and every second day*

Collections	Twice daily	Daily	Every 2nd day
Total flies	284	148	247
% females	81	60	68
Trap-days	120	120	274
Flies/trap/day	2.39	1.23	0.90
Comparison with daily visits (%)	194	100	73

TABLE 3

Total catches of Glossina palpalis *taken during an 8-week period of March to May 1960 from traps visited daily, three times weekly and twice wekly*

Collections	Visits to traps		
	daily	3/week	2/week
Males	40	33	15
Females	72	40	27
Total	112	73	42
Comparison with daily visits	100	65.2	37.5

The three-visits-a-week group gives a figure close to, though slightly lower than, those already obtained for alternate day visits, with an escape rate of over 30%. The high escape rate with only two visits per week rules out such a routine as a practical proposition.

It is significant that an investigation in the Gold Coast by M. G. Morris,[6] who used a completely different method, showed an escape rate from these traps almost identical with that found in the present work. By treating traps with DDT, which paralyzed the flies after their entry into the cage and so checked further escape, an increase of 40% in the catches was obtained. This indicates an escape rate of just under 30%.

These experiments show that for accurate comparative sampling with traps it is essential to standardize the frequency of visits. Visits on alternate days give samples 30% lower than those taken on daily visits, whereas the sample is almost doubled with visits twice a day.

Use in Survey

A year is a very short time for biological investigations, yet the work in Liberia did show the suitability of traps for sampling *G. palpalis* populations under the conditions of forest and secondary forest prevailing there. In comparison with hand catching by local Liberians, traps had the great advantage of giving samples of a known degree of standardization, which did not vary under difficult conditions or in cases of very high or very low concentrations of tsetse. The obvious disadvantage of traps was their lack of mobility. It is necessary then to examine how this would affect their use in a general survey.

Two important points must be considered in this respect. In the first place, the outstanding feature of the well-populated areas of secondary forest in northern Liberia, where trypanosomiasis was still endemic and which had held epidemic foci in the past, was that *G. palpalis* was present at very low densities; frequently only 2 or 3 flies would be caught in a week's search. This is not an unusual feature in Gambian trypanosomiasis, in which it is well recognized that the presence of the vector tsetse in small numbers can be dangerous,[7] but it imposes the necessity for surveys to be so designed that very sparse populations of *G. palpalis* are not missed. Out of this arises the second consideration. Work in the Gold Coast[2] has shown that in sampling this tsetse the length of time of search is more important than the number of searchers (using conventional fly-collectors), particularly in revealing the presence of tsetse flies at low densities. A single day's search, even with a number of men, was quite unreliable, and a minimum of 4 days of continuous searching was found necessary to give even 90% reliability.

The question of mobility now appears in a different context. The idea of rapid surveys, with collectors moving quickly from place to place, has to be abandoned. A minimum of 4 days of continuous observation at each place is required for the results to be of value epidemiologically. It has been shown that two to four traps can sample *G. palpalis* at points of man-fly contact at least as efficiently as can a team of two collectors. Yet two men properly trained could maintain as many as forty traps in continuous operation with daily visits, thus covering a much larger area than they could manage when acting as fly-collectors. Moreover, if these surveys could be made during the drier months of the year, from October to April, their catches would give a truer picture of the actual numbers of tsetse present, since this is the time when traps are working to the greatest advantage in comparison with men.

These principles were put into practice before the writer left Liberia. A team, made up of one fully trained trap-man accompanied by a learner, who was very useful in helping to carry and set up the traps, worked in pairs at 8 to 10 different places and was able to operate sixteen to twenty traps. The whole outfit could be moved from one locality to another in a Land Rover with roof-rack. It took a day setting the traps in place and a day collecting them, so that 5 days continuous sampling could be made at this number of places per week. Besides tsetse flies, the traps took a number of species of *Tabanus*, *Haematopota*, *Chrysops* and allied genera among the Tabanidae, indicating the possibilities of this method of survey in wider fields than that of African sleeping sickness.

SUMMARY

Under the conditions of tropical rain forest and secondary forest in Liberia it was found that surveys of the tsetse fly *Glossina palpalis*, the vector of Gambian sleeping sickness, could be

more efficiently carried out by traps than by the conventional method of hand catching with fly-collectors.

Unbaited traps, 2 feet high and 2 feet long, covered with natural burlap, were adopted because they gave a uniform rate of sampling. Traps twice as large caught more tsetse but were difficult to standardize. Folding traps, more easily carried than the rigid standard type, were equally effective in a short trial.

In direct comparisons, four traps caught as well as two collectors in low densities of *G. palpalis*, with a marked superiority during the dry season, when their catches gave a truer index of the numbers of tsetse present. In high densities of this species, traps gave a better performance than men, since their rate of catching was unaffected by the numbers of tsetse to be caught. It is economical to employ traps, but not men, for detecting flies at very low densities.

Traps can be used to measure the factor "man-fly contact." Comparability requires a standard procedure in visiting traps. The catches obtained with daily visits are nearly doubled with visits twice a day, and fall to 70% with visits on alternate days and to below 40% when visited twice a week. These differences are due both to the escape of flies between visits and to the attraction of tsetse to the traps by hosts each time a trap is visited.

These traps could be used also for surveys of tabanids and other insects.

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METABOLIC ALKALOSIS, A LATE COMPLICATION IN ASIATIC CHOLERA: A CASE REPORT

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Because of large fluid and electrolyte losses in the diarrheic stools during the acute phase of cholera, serious renal and metabolic disorders may result, producing leading and fatal complications. These were frequently reported in the past as uremia and acidosis[1-3] and more recently as acute tubular necrosis (due to prolonged shock and hemoconcentration) with hypokalemic nephropathy.[4]

According to the detailed studies in 1958 on fluid and electrolytes in Asiatic cholera, tremendous amounts of bicarbonate, sodium, and potassium were lost in the stools.[5] The metabolic acidosis which developed in severe and untreated cases could be explained in part by the loss of bicarbonate. Metabolic alkalosis occurring during the recovery phase of acute tubular necrosis is uncommon, and according to the available literature this condition has not previously been reported in cholera.

Case no. 8440-59. A 38-year-old Thai housewife was hospitalized 2.5 hours after acute onset of watery diarrhea, severe dehydration, and painful muscular cramps of the calves. Blood pressure was 100/70 mm Hg. Initial serum sodium was 135.4 mEq/L; potassium, 4.5 mEq/L; chloride, 112.6 mEq/L; bicarbonate, 5 mEq/L; calcium, 11 mg/100 ml; and blood NPN, 32 mg/100 ml. The stool culture was positive for V. Cholera (Ogawa serotype). The patient was rehydrated promptly with 4 liters of intravenous normal saline solution and acidosis was corrected with 2.5 liters of M/6 sodium lactate solution. No potassium was given orally or parenterally. The patient continued to pass large amounts of stools and went into shock 2 or 3 times on the 2nd and 3rd days after admission. Hematocrit ranged between 49 and 42; whole blood specific gravity,[6] between 1.065 and 1.060. Diarrhea lasted 6 days; only a few milliliters of urine was passed during the first 4 days. On the 5th day of hospitalization, her total urine output was estimated at 200 ml with 1^+ albumin, specific gravity of 1.010, and with cells and casts. Urine volume progressively increased to 1,600 ml/day with 0.56 mEq/L of urinary sodium and 15.54 mEq/L of potassium on the 8th day. The hematocrit was 39%; whole blood specific gravity, 1.056; blood NPN, 72 mg/100 ml; serum sodium, 122.7 mEq/L; and potassium, 4.0 mEq/L. On the 9th day after admission she was asymptomatic and was discharged (Table 1).

She was readmitted 6 days later, with the chief complaints being weakness, marked painful rhythmic contractions of the hand and foot muscles, and dyspepsia. Physical examination revealed carpopedal spasm, anemia and pitting edema at the dorsum of both feet. Blood pressure was 140/100 mm Hg. Trousseau's sign was positive; Chvostek's sign, negative. Serum sodium was 142.1 mEq/L; potassium, 4.26 mEq/L; calcium, 9.5 mg/100 ml; chloride, 121 mEq/L; bicarbonate, 31 mEq/L; blood NPN, 20 mg/100 ml; hematocrit, 30%; and whole blood specific gravity, 1.047. The daily urine output was 1,100 ml with an acid reaction (pH 5.8), specific gravity of 1.006, and albumin trace, but with no blood cells or casts. The urine volume was definitely increased up to 2,500 ml on the 5th day after the second admission and declined to a normal limit on the 8th day. The urine pH varied between 7.5 and 7.0 during the first 5 days and then became 5.5 to 6.5 on the 8th day. Blood examination on the 4th day revealed an Hb of 9 g/100 ml; RBC, 4.05 million/cmm; WBC, 6,000/cmm.; P, 45%; L, 44%; and E, 11%. Arterial blood pH during alkalosis ranged between 7.6 and 7.8.

While in the ward, the patient received a normal diet and had an unrestricted water intake. The painful muscular cramps during alkalosis were temporarily combatted with 10 to 20 ml of 10% calcium gluconate solution and bag breathing. No intravenous fluids were given. The patient was responsive and free from any symptoms within a week. No other specific renal func-

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TABLE 1

Observations during the patients' first period in hospital

Day	Blood pressure	Intake				Output				NPN mg%	HCO_3^- mEq/L	Cl^- mEq/L	Na^+ mEq/L	K^+ mEq/L	Hct %	Sp gr blood
		H_2O (ml)	NSS (ml)	Lactate (ml)	Total (ml)	Urine (ml)	Stool (ml)	Vomitus (ml)	Total (ml)							
1	100/70	1500	4000	2500	8000	—	4000	1000	5000	32	5	112.6	135.4	4.47	—	—
2	—*	1500	5000	—	6500	—	4500	400	4900	46	17	118.0	130.7	4.0	49	1.065
3	82/70	200	5000	—	5200	—	2900	300	3200	—	14	—	136.0	4.0	49	1.063
4	120/100	2000	—	2000	4000	—	3600	—	3600	57	10	—	133.0	4.15	43	1.059
5	100/70	1000	—	—	1000	200	500	—	700	—	25	—	—	—	—	—
6	—	1000	—	—	1000	1200	500	—	1700	72	—	112.6	133.0	3.43	—	—
7	—	1900	—	—	1900	700	—	—	700	72	—	—	122.7	4.0	39	1.056
8	—†	—	—	—	—	1600	—	—	1600	50	—	107.0	—	—	—	—

* Patient in shock.

† Urinary Na 0.56 mEq/L; K 15.6; sp gr 1.010; albumin trace, cells and casts.

TABLE 2

Observations during the patients' second period in hospital

Day	Blood pressure	Intake* (ml)	Output* (ml)	NPN mg%	HCO_3^- mEq/L	Cl^- mEq/L	Na^+ mEq/L	K^+ mEq/L	Ca^{++} mg%	Hct %	Sp gr blood	Other findings†
1	140/100	850	1100	20	31	121.0	142.1	4.26	9.5	30	1.047	U.pH 5.8; sp.gr 1.006; T^+
2	—	860	1500	20	27	118.0	—	—	9.0	—	—	U.pH 7.5; HCO_3^- 4 mEq/L; Ca 7.6 mg%
3	100/70	500	2250	20	32	115.5	137.6	4.9	9.6	—	—	U.pH 7.0; B.pH 7.6; T^+
4	—	900	2300	—	33	—	137.0	4.8	—	28	1.047	U.pH 7.0; HCO_3^- 2 mEq/L; B.pH 7.82
5	—	1000	2500	20	31	126.7	134.5	4.9	8.3	—	—	U.pH 7.0; HCO_3^- 4 mEq/L; T^-
6	—	850	2000	—	—	—	—	—	—	—	—	U.pH 5.5
7	—	750	1800	—	—	—	—	—	—	—	—	U. sp gr 1.005
8	—	950	1750	—	—	—	—	—	—	—	—	
9	—	700	900	20	—	115.5	135.1	5.0	9.0	—	—	U.pH 6.5; B.pH 7.4; T^-
10	—	1150	1100	—	—	—	—	—	—	—	—	
11	—	1500	1700	—	—	—	—	—	—	—	—	
12	—	1050	2100	—	—	—	—	—	—	—	—	
13	—	1100	1600	—	—	—	—	—	—	—	—	
14	—	1300	1700	—	—	—	—	—	—	—	—	
15	—	1150	2100	—	—	—	—	—	—	—	—	
16	—	900	1700	—	—	—	—	—	—	—	—	
17	—	1450	1500	—	—	—	—	—	—	—	—	
18	—	1300	1400	—	—	—	—	—	—	—	—	

* Water constituted the total intake, urine the total output.

† U.pH = urine pH; B.pH = blood pH; T = Trousseau's sign.

tion studies were performed. She was discharged 18 days after the second admission and when seen again 1 month later was in normal health (Table 2).

Comment. This patient had definite clinical features of mild acute renal tubular necrosis. The anuric phase was not observed carefully and she might have had a continuation of the diuretic phase into the second admission. This late polyuria might also have resulted from hypokalemic changes in the kidneys.[4]

During the acute phase of cholera one might lose as much as 33.3 mEq/L of potassium in the stools with total losses estimated as high as 700 mEq of body potassium.[5] A markedly reduced total body potassium, even though serum potassium was within normal limits, was confirmed by definite pathologic features of hypokalemic nephropathy as reported by Benyajati *et al.* in 1960.[4] Apart from potassium loss, acidosis which was due mainly to the loss of bicarbonate in the stools, was adequately corrected during this patient's first admission. In alkalotic patients with hypokalemia and in animal experiments,[7] renal tubular reabsorption of bicarbonate was increased. This reabsorption was inhibited by carbonic anhydrase inhibitors as well as by potassium in human and animal studies.[7, 8] The pathology incurred in acute tubular necrosis and/or marked potassium loss (presumably without bicarbonate loss) provide reasonable explanations for the abnormalities of renal tubular functions which were seen in association with metabolic alkalosis and tetany. The acid urine was also explainable as the paradoxical aciduria common in other potassium deficiency alkalotic cases.

SUMMARY

A case of cholera with acute tubular necrosis and metabolic alkalosis is reported. The etiology and mechanisms of metabolic alkalosis are discussed.

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BLADDER STONE IN THAILAND. A REVIEW OF THE PROBLEM

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Bladder stone in children was a familiar condition in Europe and the United States[1, 2] until the beginning of the 20th century and is still a major problem in areas of Southeast Asia, India and the Middle East.[3-6] This fact was brought home to the author during a recent visit to Northeast Thailand where during 1959 over one-half of the operations performed by the surgical service of Ubon Provincial Hospital, a 200-bed hospital in the area, were for bladder stone. In this hospital it was not uncommon to have 5 or more lithotomies scheduled per day. Although bladder stone in Thailand has received considerable attention by Thai physicians, only two references in English are found in the recent literature[3, 7]. The purpose of this article is to review the problem in Thailand, to discuss present day thinking regarding its etiology, and to outline studies which might further understanding of this condition.

Thailand, geography and people. Thailand is about as large as France, lying entirely within the northern tropical zone in the center of the Indochinese peninsula. The country can be divided into 4 regions (Figure 1): (1) Central Thailand or the Central Plain is formed by the alluvial basin of Thailand's major river, the Chao Phraya; (2) Northeast Thailand consists of the Khorat plateau, a moderately hilly land averaging 200 feet above sea level and bounded on the east by the Mekong river; (3) North Thailand is a region of long north-south mountain ridges and deep narrow alluvial valleys; and (4) South Thailand is a narrow, rather mountainous portion of the Malay peninsula. The Central Plain is a rich rice growing region which contains 40% of Thailand's 21 million people. The Northeast has fine sandy loam soils low in plant nutrients and easily overworked making agriculture in this region an arduous and somewhat unproductive occupation. About one-quarter of the Thai population lives in this area. The North with 15% of the population has clay soils which support wet rice cultivation. The South has fairly poor food soils, but the land supports rubber trees and yields tin so that agriculture is relatively unimportant.[8]

The Thais are racially homogeneous; however, there are moderate ethnic and linguistic differences between the peoples of the natural regions.[9] About 3 million Chinese live in Thailand, mainly in urban areas, concerning themselves with the trades and business. The Chinese retain a community identity which includes a distinctive diet and methods of food preparation which differ from those of Thai. Thus the Chinese enjoy both a pattern of life and nutritional status different from the Thais living in the same areas.[10]

Bladder stone—geographical distribution and prevalence. Recently, a compilation of provincial hospital admission statistics in Thailand by Sanong has provided the first objective evidence that bladder stone is limited to certain geographic areas of Thailand.[11] The distribution of hospitalized urolithiasis based upon these statistics for all Thailand in 1956 is shown in Figure 2. During 1956 there were 3,797 patients with urinary calculi admitted to provincial hospitals, 91.2% of which were bladder or urethral stones. The admission figures expressed as hospitalizations per 100,000 population point to the existence of two endemic foci, the Northeast and the North, with low rates prevailing in the Central Plain and South Thailand. The degree to which hospital admissions reflect the residence of patients is not known. The apparent contradiction found in the relatively high rates observed in Bangkok, outside the endemic area, can probably be explained by the fact that the superior medical facilities of this city attract patients from long distances. This may also be true for the cities of Chiengmai and Songkhla which may attract enough patients from surrounding provinces to raise their provincial rates above those of their neighbors (Figures 1 and 2).

In some areas of Thailand the magnitude of the problem is enormous. During 23 years association with the Ubon Provincial Hospital, Chutikom has studied nearly 7,000 cases of bladder stone.[12] Thousands of stones collected over the past 30

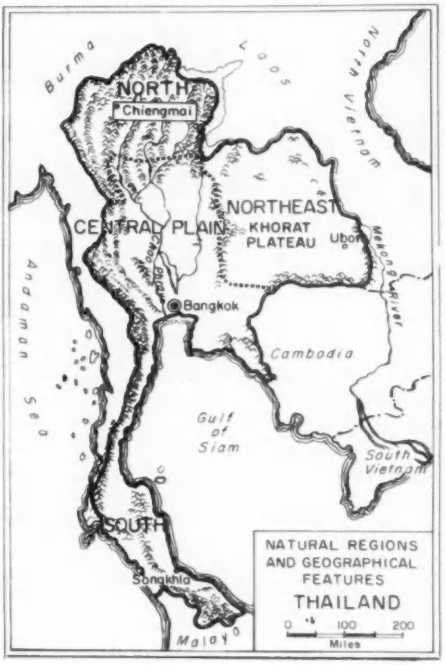

FIG. 1. The natural regions, geographical features and major cities of Thailand. After Blanchard.[8]

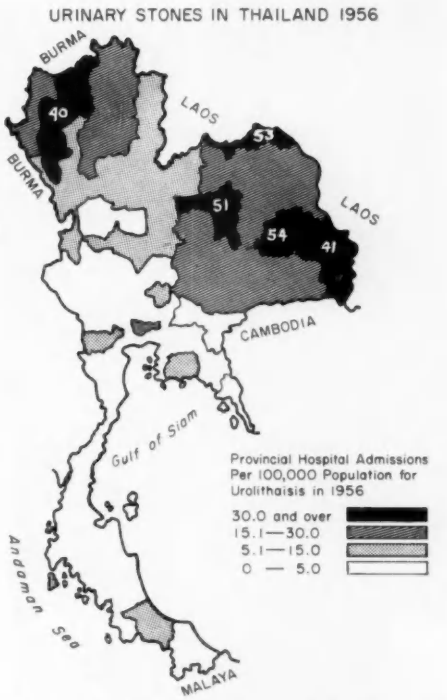

FIG. 2. Incidence of hospitalized urolithiasis in Thailand for 1956 (91.2% bladder or urethral stones). Figures for the 5 provinces with highest incidence are indicated. After Sanong.[11]

years are on view in the hospital museum (figures 3–5) and attest to the frequency of this condition. In 1951 in a village near Ubon, Passmore found that 1 in every 125 persons either had stone or had a history of stone.[3] In Chiengmai, North Thailand, in the years 1955 to 1958 between 30 and 40% of all non-obstetrical and non-gynecological operative procedures were operations for vesical calculus.[13]

Prevalence of Bladder Stone with Respect to Certain Factors

Age. Bladder stone in Thailand is a disease of children. In the approximately 7,000 cases studied by Chutikom the median age at operation is between 4 and 5 years.[12] This finding is consistent with earlier data collected at the same hospital and reported by Passmore.[3] His findings are illustrated in Figure 6 which shows both male and female operation rates per 10,000 population with a peak incidence for both sexes at about 5 years of age. Several children in this series were operated on under the age of 1 year. In Passmore's report 90% of bladder stones occurred in persons under the age of 20. An increase in the 6th and 7th decade of life resembles a similar increase in bladder stone for these age groups seen in the United States. Sanong has reported the instance of an operation for bladder stones in an infant 21 days old.[11] In his series for all Thailand 61.4% of all stones occurred in persons under the age of 20. Whether this reflects delayed recognition of bladder stone in some areas of Thailand or a real difference in age of onset of symptomatic urolithiasis in different areas of the country is not known.

Sex. Bladder stone is preponderantly a disease of males. In both Sanong's and Passmore's published accounts bladder stone occurs approximately 8 times more frequently in males than in females. This is in agreement with recent data from the New Delhi area of India where the

FIG. 3. A portion of the hospital collection of bladder stones on display at the Ubon Provincial Hospital museum, Thailand.

ratio was 12:1[4] and with other major observations on bladder stone in children most of which report an even greater preponderance in males.[14]

Racial prevalence. Bladder stone in Thailand is said to occur less frequently in the Chinese than in the Thai. Chutikom knows of no cases among the Chinese inhabitants of Ubon City.[12] Sanong reported that 3% of all cases of urolithiasis were in Chinese.[11] The proportion of bladder stone or kidney stone in Chinese was not given. Since Chinese constitute approximately 15% of Thailand's population the prevalence of this condition in Chinese is lower than would be expected. It is doubtful that the Chinese are racially insusceptible, however, in light of reports of the endemicity of bladder stone in the Canton area of South China.[6] It is suggested below that bladder stone occurs infrequently in urban residents. Since Chinese are urban dwellers in Thailand the only pertinent comparison would be the prevalence of bladder stone in urban Thais and in Chinese. Without this data no conclusions can be reached concerning racial susceptibility to bladder stone in Thailand.

Family prevalence. Some observers believe that the family incidence of stone is high. Chutikom states that 25% of the patients in his series gave a history of a family member suffering from stone.[12] This statement is difficult to assess without further data; supporting evidence awaits future epidemiologic studies.

Rural vs. Urban Prevalence. Preliminary information suggests that bladder stone spares urban inhabitants living in endemic areas. Chutikom has stated that he has never seen a case of bladder stone occur among residents of Ubon, a city of 25,000 persons and the only urban area of any size in Ubon Province.[12] Passmore reported high prevalence of the disease in small rural communities near Ubon city.[3]

Recurrence. Compared with a common form of urolithiasis in the United States the recurrence rate of bladder stone in Thailand is low. In Ubon less than 1% of the patients were said to have recurrence.[12] This is in patients who are returned to their original environment and diet. Recurrence rates for bladder stone in the United States are generally not available, although figures from 5 large series in the United States for post-operative recurrence of kidney stone in the era before dietary therapy are cited by Higgins.[15] Recurrence rates varied between 10.7 and 56%; the majority of series cited reported rates of greater than 25%. These differences in rates of recurrence add to the evidence which suggests that vesical lithiasis in Thailand and nephrolithiasis in the United States are distinct entities. It is not known whether predisposing factors such as bladder infection contribute to the rate of recurrence of bladder stone in Thailand, or whether recurrence occurs *de novo* at approximately the rate of first attacks.

Composition of stones. Sanong and others have analyzed hundreds of stones sent from all over Thailand and have observed that nearly every salt commonly found in urinary stones can be demonstrated. Ammonium urate, calcium oxalate and calcium phosphates predominate, but uric acid and calcium carbonate are also found.[16, 17] Individual stones may be of relatively pure composition, but stones from the same locality show a variation of salts. Thus, it did not appear

FIG. 4. Bladder stones showing variation in size, shape and color

FIG. 5. Two large bladder stones are examined by Dr. Cholvit Chutikom, Hospital Director, Ubon Provincial Hospital.

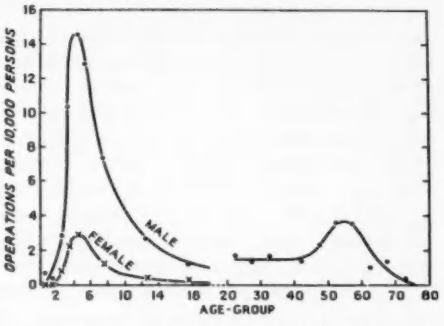

FIG. 6. Age and sex distribution of bladder stone at Ubon Hospital, 1947–1951. After Passmore.[3]

that the salt deposited in the stone was related to geographic area. Extension of these studies to a representative sample of stones from all over Thailand would be valuable especially if the composition of the "kernel" of the stone were differentiated from salts deposited after initial formation.

DISCUSSION

The mechanism of the formation of urinary stones is still poorly understood. Major studies

of urinary calculus formation have been made in the United States and Europe where renal and bladder stones occur predominantly in adults and are studied collectively. Extrapolation of information derived from these sources to the pathogenesis of bladder stone in Thailand is hazardous, but is the only theoretical framework available at present. As is pointed out in the excellent reviews of McGeown and others,[18-20] the normal noncalculus state is a result of a balance of factors which tend to increase or decrease the solubility of normally insoluble salts in the urine and the ability of the kidney and the genito-urinary (GU) tract to rid itself of small precipitates as they are formed. It has long been observed that normal urine is markedly hypersoluble for excreted crystalloids even resisting to a marked degree precipitation in superconcentrated solutions produced during dehydration. Hypersolubility has been explained to some extent by observations that solubility of precipitable salts varies with pH[18] and is directly related to the concentration of some organic and inorganic solutes such as urea,[21] certain amino and organic acids,[18] citrates[22] and some urinary colloids.[23] Other colloid substances found in the urine may actually enhance the precipitation of calcium salts and serve as a matrix to organize the growth of crystals in stones.[18, 24] Resemblances between the matrices of bone and urinary stones have led to the suggestion that stone growth may be an active process controlled by unknown factors but not dependant directly upon the concentration of precipitable salt in the urine.[24] It has been suggested that calcium deposits in the kidney[25] or keratinized GU tract epithelium[26] may provide a nidus for stone growth. Surprisingly, excess ingestion of precursors of insoluble urinary salts has rarely been implicated as a primary cause of urolithiasis.[27] From the multiplicity of mechanisms and theories of urolithiasis which are current, it seems reasonable to suppose that there is no single explanation for the formation of urinary calculi. On the other hand, endemic urolithiasis does support belief in a common pathogenetic mechanism. It is quite possible that one of the mechanisms briefly alluded to above may be of central importance in explaining bladder stone in Thailand.

Specific predisposing conditions associated with vesical calculi have been reviewed recently by Ramalingaswami and Aurora.[4] The list of known conditions resulting in bladder stone includes congenital anomalies of the bladder and urethra, bladder infection, obstruction of the bladder neck, urinary stasis and foreign bodies in the baldder. Known causes of urinary stones in the lower as well as the upper urinary tract include the errors of metabolism of cystinuria, familial oxaluria, xanthinuria, and gout; and conditions producing hypercalcinuria such as hyperparathyroidism, Cushing's disease, milk-alkali syndrome, excess vitamin D intake and renal tubular acidosis. None of these conditions satisfactorily explains bladder stone in Thailand. The marked endemicity and focal distribution of bladder stone, the preponderance of stones in young males and the reported low recurrence rate would be most unusual for any of the conditions cited.

With the present limitation to our knowledge of the pathogenesis of urolithiasis, it has been necessary to rely on epidemiologic evidence to derive an hypothesis for the cause of bladder stone. In his classic Ramon Guiteras Lecture in 1934, Joly summarized the overwhelming weight of evidence which implicates nutritional abnormality (excess or deficiency) as the external factor responsible for bladder stone of children.[27] Three types of supporting studies in laboratory animals have followed. (1) *Vitamin A deficiency.* Rats deprived of vitamin A and fed a high calcium diet will regularly develop urolithiasis.[25] Vitamin A deficiency results in keratinization of the epithelium of the urinary tract which is thought to provide a nidus for stone growth.[24] (2) *Magnesium deficiency.* Hammarsten has shown that rats maintained on a diet deficient in vitamin A and magnesium had increased excretion of urinary calcium with the formation of calcium oxalate stone in low pH urine, and calcium phosphate stone in urine of high pH.[28] Restriction of calcium and oxalates in the diet did not alter the results. These studies have been confirmed by Prien who has shown that magnesium deficiency alone is sufficient to produce calculi.[29] (3) *Vitamin B_6 Deficiency.* Recently, Andrus and others have demonstrated calcium oxalate stone formation in pyridoxine deficient rats.[30] As yet a definite relationship between nutrition and bladder stone has never been proven for humans although Joly and others have made a strong case for the etiologic role of vitamin A deficiency. However, the widespread distribution of deficiency diseases in Southeast Asia and India con-

trasted with the focal occurrence of bladder stone in these countries has presented a formidable obstacle to the acceptance of this hypothesis.

In Thailand there are two observations which lead one to believe that some nutritional factor or factors are important in bladder stone formation: (1) the high incidence of the disease in regions of the country which are geographically distinct and in which the type of soil, mode of agriculture and diet differs in some respects from the non-stone producing regions; and (2) the reported disparity in incidence of the disease between rural inhabitants and townspeople of the Northeast.

The hypothesis that vitamin A deficiency is this dietary factor receives both contradiction and support from Thailand. (1) *Vitamin A deficiencies are seen in all parts of Thailand.* However, bladder stone occurs mainly in limited areas of the country. In spite of some regional differences the basic components of Thai food and practices of food preparation are common to most of Thailand and provide a diet poor in vitamin A content. In South and Central Thailand rice together with salt water fish and in North Thailand rice and fresh water fish are staples; in the Northeast the staples are glutinous rice and "fermented" fresh water fish. Polished rice is preferred and vegetables and rice are cooked in excess water which is discarded before eating. It is not surprising that severe vitamin A deficiencies are still commonly seen in infants in all sections of the country or that all Thai diets are considered low in vitamin A content.[31] Yet some of these areas are almost entirely free of bladder stone. (2) *The degree of vitamin A deficiency may be greater in the stone forming regions than in other areas of Thailand.* Nutritional surveys have shown a greater deficiency in caloric and vitamin content of diets in North and Northeast Thailand when compared with those of South and Central Thailand.[31, 32]

There are interesting regional dietary variations in Thailand which may be of importance in bladder stone disease. For example, in the North and Northeast, seed parts and leaves from forest plants are regularly consumed as vegetables. A crude salt made in the Northeast by evaporating soil and water suspensions is widely used and marketed. Glutinous rice is a food staple in much of the Northeast and North. Finally, raw fresh water fish consumption has already been associated with Opisthorchiasis, a disease endemic in Northeast and North Thailand.[33] Unfortunately, the nutritional aspects of these and other regional variations are at present insufficiently studied to add much to hypotheses of bladder stone formation.

Without any obvious etiology at hand for bladder stone in Thailand, one is led to speculate from the salient epidemiologic features of the disease presented above.

Bladder stone is a disease of the young. The age distribution of cases suggests a disease in a group exposed to a causative factor during an early period of life. The resultant stone matures during an "incubation period" before the onset of symptoms. It is reasonable to suppose that the initial formation of stone occurs during the first year of life or possibly prenatally. Once formed, further growth proceeds at different rates depending upon individual factors. Variation in "incubation period" may result from the fact that most symptoms requiring hospitalization for bladder stone are not dependent entirely on stone size or duration of the stone in the bladder, *i.e.*, dysuria and acute urinary retention are related to impaction of any size or age stone in the bladder neck; symptoms of urinary tract infection may intervene at any stage of calculous disease. Initial stone formation may well be the result of a relatively acute deficiency or excess of factors never again duplicated in childhood. This hypothesis would account for the observation that bladder stone recurrence is extremely infrequent when compared to other forms of urolithiasis.

It is the custom in Thailand to nurse children up to 2 years of age or more. After approximately 1 year, mother's milk is supplemented with rice water and other fluids. The child is then gradually introduced to adult foods. The primary source of nourishment then, during the first years of life in Thailand, is human milk—a fact which suggestively points a postulated nutritional abnormality to the mother and which would imply that the stone forming factor or deficiency is one which is transmissible in milk or perhaps prenatally. A hypothetical stone producing factor might actually be concentrated in milk.

The sexual difference in incidence of stone in the bladder is of interest. Unless females are shown to be metabolically less susceptible to this disease, this observation stresses the importance of the structure of the genito-urinary tract in

stone formation. Presumably the narrower bladder neck or tendency for sphincter spasm in the male[34] contributes to the retention of small stones in the bladder allowing further growth to take place.

There can be little doubt that a subclinical state exists in bladder stone disease. This does not refer to an asymptomatic child with a well formed calculus, but rather to a child which passes all urinary precipitates out of the bladder in the form of gravel or still smaller particles. In stone forming areas the frequency of subclinical vesical lithiasis may be very great. The demonstration of this state is vital to establish the true prevalence of the biochemical defect associated this disease.

The possibility that an ingested "positive noxious factor" exists which causes bladder stone has been forwarded by Passmore.[3] He postulated that a plant poison might interfere with the colloid mechanism or with citric acid excretion somewhat in the manner that potassium fluoracetate found in the South African plant *Sichapatalum cymosum* blocks the excretion of citric acid by the kidney. Citric acid has been shown by Shorr and others,[22] to "enhance calcium solubility" in the urine. Such a factor might be contained in the leaf and seed parts of forest plants consumed in Northeast Thailand. Whether dietary customs and plant speciation are similar in North Thailand is crucial to the retention of this hypothesis. The reported difference in incidence of bladder stone between townspeople and rural inhabitants of the Northeast need not contradict this hypothesis since town dwellers have access to a restricted number of forest plants for consumption; furthermore, the diet of townspeople is supplemented with products from other parts of Thailand. It is possible that the action of a plant "stone-forming factor" may depend upon a chain of events. For example, such a substance may be produced in a limited number of plant species and only optimally under specific soil conditions and at certain seasons of the year. It may be effective in humans with relative nutritional deficiencies or when concentrated in the diet (human milk?) or in the body as in the dehydration of infantile diarrhea.

It is difficult to connect dietary vegetative peculiarities in Thailand with bladder stones in India or the Middle East or with the occurrence of stones in the recent past in Austria or Iceland.[27] An hypothesis of bladder stone formation implicating a single etiologic factor may have to be abandoned in favor of a theory which can account for regional dietary differences leaving nutritional deficiency as an important common denominator in stone pathogenesis.

Whatever the etiology of bladder stones in children in Thailand, the mechanisms of this disease should be amenable to further clarification by modern biochemical and epidemiologic techniques. A concerted effort to solve the riddle of bladder stone might well begin in Thailand where the health of thousands of children is jeopardized by this capricious disease.

SUMMARY

In Thailand, bladder stone is a highly endemic disease in children in two of four distinct geographic regions of the country, the North and Northeast. Vesical stones occur early in life; a median age of 5 years has been reported in Ubon Province, and they are seen 8 times more frequently in males than in females. Preliminary observations suggest that bladder stones are more common in rural than in urban areas and that recurrence rates are low (less than 1%). No adequate hypothesis for the pathogenesis of bladder stone exists, although extended observations of this condition in Thailand and elsewhere strongly implicate a deficiency or excess of some human nutritional factor(s). The early age of onset of bladder stone suggests the possibility of the transmission of a lithogenic factor in human milk. This hypothesis may serve as a departure for the additional epidemiological and clinical studies which are needed to clarify our understanding of this problem.

ACKNOWLEDGMENTS

Without the willing and gracious co-operation of Dr. Cholvit Chutikom, director of the Ubon Provincial Hospital, Ubon, Thailand, and Prof. Unakul Sanong, Division of Biochemistry, Department of Physiology at Siriraj Medical School, Dhonburi, Thailand, this enquiry would not have begun. Special thanks go to Dr. Ross L. Gauld for his helpful suggestions in the preparation of the manuscript.

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BOOKS RECEIVED

Listeriosis, by Privatdozent Dr. med. H. P. R. Seeliger, Hygiene - Institut, Rhenische Friedrich - Wilhelms - Universität, Bonn Rh. (Germany; foreword by Dr. Dr. h. c. K. F. Meyer, Hooper Foundation for Medical Research, San Francisco, California; with an introduction to the 2nd German edition by Prof. Dr. Dr. H. Eyer, o. ö. Professor für Hygiene und Medizinische Mikrobiologie, Max v. Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie der Ludwig-Maximilians - Universität, München (Germany). 308 pages, illustrated (67 illustrations, 5 of them colored). New York, Hafner Publishing Company, Inc., 1961. $14.25.

The Chemotherapy of Tropical Diseases, by Sit Philip Manson - Bahr, M.D., F.R.C.P, D.T.M.&H. (Cantab), Consulting Physician to the Hospital for Tropical Diseases, London, and John H. Walters, M.D., F.R.C.P., Lt. Colonel I.M.S. (ret.), Physician, The Hospital for Tropical Diseases, London. 164 pages + xi pages. Springfield, Illinois Charles C Thomas, Publisher 1961. $7.00.

Bone Changes in Leprosy, by Vilh. Møller-Christensen, M.D.; Translated from the Danish by Anita Engelbreth-Holm. 51 pages, illustrated, paper covers. Copenhagen, Denmark, Munksgaard, 1961. Price Dan. Kr. 32.–.

BOOK REVIEWS

Manson's Tropical Diseases. A Manual of the Diseases of Warm Climates, Sir Philip H. Manson-Bahr, C.M.G., D.S.O., M.A., M.D., D.T.M. and H. Cantab., F.R.C.P. Lond., M.D. (Malaya-Hon. Causa), Editor. 15th edition, 1177 + xiv pages, illustrated. London, Cassell (The Williams & Wilkins Co., Baltimore, exclusive U.S. agents), 1960. $15.00.

This text has become a classic reference in tropical medicine in the English language. It was first published in 1898 and the present 15th edition by Sir Philip H. Manson-Bahr is the ninth in his experience with this book. The previous edition appeared in 1954 and in the interval between then and the present edition considerable progress has been made in a number of clinical areas in the field of parasitism and tropical medicine, particularly the anemias, leprosy, insecticides and chemotherapy. In these various areas major revisions and additions have been incorporated in the text and the previous material which has been basically excellent has been revised wherever indicated. The table of drugs, particularly in Chapter LIII, is extremely up to date and a very valuable and convenient reference. The sections on Medical Zoology in the Appendix contains more than 200 pages and can easily serve as a useful text in medical parasitology, including entomology, for students. On the whole this is a most gratifying book to have in one's possession; it is well written, nicely printed and arranged and profusely illustrated with useful tables, graphs, photographs and drawings.

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Animal Parasites in Man, by N. H. Swellengrebel, Ph.D., M.D., The Royal Tropical Institute, Amsterdam, and M. M. Sterman, M.D., Public Health Physician, Tropical Disease Diagnostic Service, Bureau of Preventable Diseases, New York City Department of Health, Instructor in Tropical Diseases, College of Physicians and Surgeons, Columbia University, Associate Physician in Tropical Diseases and Parasitology, Knickerbocker and Elmhurst General Hospitals. 652 + x pages, illustrated. Princeton, New Jersey, Toronto, London, and New York, D. Van Nostrand Company, Inc., 1961. $12.00.

According to the publisher, this book provides a full account of "the life cycles and morphology of protozoa, helminths, insects, and other arthropod parasites that afflict mankind" and is designed especially for biologists and medical doctors.

The title, "Animal Parasites in Man" which uses "animal" to qualify parasites is a definite step forward in exact terminology, for the present trend in microbiology is to designate all microorganisms that cause disease as "parasites"

whether they be viral, bacterial, or animal in nature. It has been common practice in the past to limit the word parasite to animals, and it is hoped that the current accurate designation marks a significant change in usage which will be accepted by all microbiologists including animal parasitologists.

The descriptions of the animal parasites and their life cycles in the text are concise and for the most part adequate. They likewise usually portray up-to-the-minute discussions on theoretical and controversial aspects of each infection. Much of the book is a translation of an earlier text in Dutch by the senior author, Dr. Swellengrebel. His presentation is refreshing when compared with the ponderous style of many textbook writers. In fact the subject matter is presented in the characteristically clear and stimulating manner familiar to those friends of Dr. Swellengrebel who have been fortunate enough to hear him lecture and speak at scientific meetings.

The subject matter in the text is presented from the viewpoint of a biologist rather than that of a clinician. The life cycles and methods of transmission of the parasites are on the whole well described but their application to public health is not emphasized. Although up-to-date procedures for treatment of disease are included in each chapter, the signs and symptoms, the pathology produced, and methods of prevention are not summarized in a manner convenient for medical students and physicians.

A separate section on arthropods important to disease together with a chapter on insecticides and repellents provides valuable information for academic students in biology as well as for medical students. Illustrations in this section are well chosen to present the subject to students who desire a general knowledge of the field rather than to those requiring specialization.

Procedures for laboratory diagnosis are presented in a separate part entitled, "Technical Methods and Diagnostic Procedures," but this part is so far removed from information regarding the morphologic characteristics of animal parasites described earlier in the book and which are necessary for their differentiation, that much of their significance is lost. Illustrations of the helminths in the main text are for the most part satisfactory, but unfortunately those of the intestinal protozoa are diagrammatic and fail to provide the detail necessary for clear cytologic differentiation.

It is the impression of the reviewer that this publication provides an excellent reference work for classes in animal parasitology both in academic and medical fields.

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The Rickettsial Diseases, by P. F. ZDRODOVSKII AND H. M. GOLINEVICH; Translated from the Russian by B. HAIGH, M.A., M.B., B.Chir., Late Physician to H.B.M. Embassy, Moscow, U.S.S.R. 629 + xii pages, illustrated. New York, Oxford, London, and Paris, Pergamon Press, 1960. $17.50.

There is a need for a comprehensive treatise on the rickettsial diseases of man or on the rickettsiae in general, accumulating and correlating the information of the past generation or so. This pedantic and tedious book does not fill this need. The fact that it is a translation perhaps excuses some of its shortcomings, but the text so commonly contains statements such as "as has been repeatedly pointed out" and "as has already been stated" that the authors themselves must have been aware of the repetitious character of their work. The book is divided into two parts, the first concerning morphologic and laboratory aspects of the rickettsiae in general and the second with the specific diseases caused by these agents. There is considerable unnecessary overlapping between the two, and at the end of the book there are five chapters of a more general nature which would have fitted more naturally into the first section.

This work is of value in that it describes and gives reference to a mass of the Russian literature on the rickettsial diseases covering the past 20 years, much of which is not readily available. It was of great interest, for example, to read that Russian investigators had grown the agent of Wolhynian (Trench) Fever "in a special nutrient medium which we call KZhM." However, no further details are given in the book and the text reference is not to be found in the bibliography, thus leaving the investigator eager to confirm this important observation without any possible means of doing so.

The book is profusely illustrated with sketches, colored drawings, photomicrographs and temperature charts. Some of these are of good quality, particularly the colored drawings and photographs of human skin lesions. There are, however, 48 photomicrographs of rickettsiae of every conceivable species in a variety of tissues and media, most of which look very much alike. Five high quality photomicrographs would have illustrated

the salient features of rickettsial morphology. There are about 30 temperature charts of guinea pigs and other animals when one tenth this number would have sufficed. There are adequate illustrations of the arthropod vectors of the various diseases, and a brief chapter on arthropods is one of the best in the book. A chapter on the mammalian hosts involved in the ecology of some of the important rickettsial diseases is conspiciously lacking.

To the non-Russian reader, the most interesting and best written chapters are those concerned with North Asian tick-borne rickettsiosis and other tick-borne rickettsial diseases, an area where the Soviet scientists have apparently done a good deal of useful investigation. One of the more abysmal chapters is that on rickettsialpox in which the authors elect to describe in the first 11 pages what they have termed vesicular rickettsiosis and what the Soviet findings have been in this field; a final page and a half are devoted to "the Rickettsialpox of the U.S.A." and the prior investigations of Huebner and others together with the statement: "probably the two diseases are identical." Throughout the book there is an unfortunate tendency to separate every topic and investigation into (a) Soviet and (b) foreign studies. This results in constant repetition and artificial division rather than a unified treatise on each topic which would usefully serve the interested reader.

In a book of this type a good bibliography can often compensate for many other shortcomings. The references to the Russian literature probably are fairly complete and should therefore be valuable to those in the field. The bibliography on the remainder of the world's literature is poorly organized and incomplete; it is virtually impossible to go from text to bibliography on any of the more significant points. Many classic investigations are omitted completely, for example, Brill (1910) or Dyer, Rumreich and Badger (1931). In the section entitled "the survival of typhus fever virus between epidemics" the authors list five or six hypotheses and text reference a number of proponents of each. It is particularly frustrating to find references supporting the Brill-Zinsser hypothesis by Plotz (1943) or Murray and Snyder (1950) not listed in the bibliography, while the rather improbable findings of isolations of *Rickettsia prowazeki* from goats and sheep by Reiss-Gutfreund in Addis Ababa are fully documented.

As a source of reference to Soviet investigations on rickettsial diseases, this book belongs on the bookshelves of a laboratory actively working in this field. It has limited usefulness elsewhere.

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Listeriosis, by Privatdozent Dr. med. H. P. R. SEELIGER, Hygiene-Institut, Rheinische Friedrich-Wilhelms-Universität, Bonn/Rh. (Germany); foreword by Dr. Dr. h. c. K. F. MEYER, Hooper Foundation for Medical Research, San Francisco, California; with an introduction to the 2nd German edition by Prof. Dr. Dr. H. EYER, o. ö. Professor für Hygiene und Medizinische Mikrobiologie, Max v. Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie der Ludwig-Maximilians-Universität, München (Germany). 308 pages, illustrated (67 illustrations, 5 of them colored). New York, Hafner Publishing Company, Inc., 1961. $14.25.

Listeriosis has captured the interest of many persons throughout the world who are engaged in either academic or clinical medicine.

A comprehensive review of this subject was presented in a German monograph *Listeriose* by Dr. Heinz Seeliger, published in Leipzig in 1955. A second German edition of this work was brought out in 1957. The present volume *Listeriosis* by the same author is especially welcome as it appears in English. The newer information has been added to bring it up to date. Additional illustrations and rearrangements of certain tables have added clarity to the original work.

The material is presented in the four sections that proved very usable in the earlier editions.

Section A describes in detail the causative agent. This is particularly valuable for the research worker.

Section B discusses listeriosis in animals. This is of interest to all but primarily so to the veterinarians.

Section C gves a comprehensive discussion of listeriosis in man.

The various types of human listeriosis are presented according to the localization of the organism in the body. This has become known as the Seeliger Classification of Listeriosis.

This makes pertinant material available to doctors engaged in the various medical specialities: internal medicine, ophthalmology, neurology, obstetrics, pediatrics and pathology as well as to the doctor in general practice.

Epidemology is discussed. There is still no clear understanding of transmission except in cases of pregnancy where the fetus or the infant is infected directly from the infected mother.

Under control measures special warning is given and a specific suggestion made. Human and veterinary public health officers should instruct people who come in contact with animals—breeders, keepers, farmers, veterinarians, workers in abattoirs—as to the possible danger of infection. No artificial immunization is known so prophylaxis rests on sanitary measures alone. Doctors, nurses, and midwives should be made aware of the gross

contamination present when delivering a listeric baby. It is urged that listeriosis be considered a contagious disease and the patients be isolated accordingly. As such it would be reportable.

Therapy is discussed in detail and the remedy, clinical efficiency and dosage are tabulated.

Section D is devoted to a comprehensive discussion of bacteriological and serological methods of diagnosis.

Each section is provided with a summary of the pertinent material presented.

References are given to 544 individual articles on listeriosis.

The index is sufficiently detailed so one can find the reference to any particular aspect of the subject without undo searching.

This book should do much toward bringing an understanding of this very important subject to English reading medical groups.

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Fortschritte der Geburtshilfe und Gynäkologie, A. REIST, Zürich, Editor. Volume 11, **Zum Problem der Toxoplasmose.** "Die Laboratoriumsdiagnostik der Toxoplasmose," by W. ROTH, Basel; and "The Importance of Latent Maternal Infection with Toxoplasma in Obstetrics," by JOSEF A. ČECH and OTTO JÍROVEC, Prague. 90 + iv pages, paper covers. Basel (Schweiz) and New York, S. Karger, 1961. Sfr. 17.70.

The author of the first article introduces his subject by pointing out the various manifestations of toxoplasmosis which so often make it difficult to diagnose. This is followed by descriptions of the morphology of *Toxoplasma* in stained preparations and in the living state. Behavior in laboratory animals and maintenance of strains are discussed. Methods of demonstration of the parasite from clinical material are presented; these include inoculation of laboratory animals, chicken eggs and tissue culture. The section on serologic methods deals with the rabbit neutralization test, complement-fixation test, Sabin-Feldman dye-test and the toxoplasmin skin test. Each of these is briefly described and commented upon. Interpretation of the results obtained with serologic tests is discussed in some detail, principally with reference to the dye-test and complement-fixation test. A comprehensive picture of present day knowledge concerning the epidemiology of toxoplasmosis is presented in the final section which includes comments on immunologic surveys, sources of infection and methods of transmission. Many of the more important references on this disease are included in the bibliography. This publication is recommended for obstetricians, pediatricians and others who are interested in a concise picture of our present knowledge of and diagnostic procedure for toxoplasmosis.

In the second paper the authors present their observations during the period of 1954–1956 on a group of 379 women in whom various degrees of perinatal fetal abnormality had occurred. The purpose of the study was to gather evidence which would point out the importance of latent or inapparent toxoplasma infection during pregnancy. Data on each patient consisted of detailed clinical history and examination, the usual laboratory tests, serologic tests for listeriosis and brucellosis, chest and skull x-rays, and the toxoplasmin skin test. Complete laboratory and clinical examinations of the new-born infants were carried out by a pediatrician. However, serologic tests such as the Sabin-Feldman dye-test or the complement-fixation test were not routinely used. Isolation of the toxoplasmas by animal inoculation also was not attempted.

These investigators believe that the intradermal skin test is more useful because it is simpler and specifically indicates previous infections which are no longer detectable by serologic methods. They realize that the test is limited in that it is only qualitative and also that it will not detect a recently acquired infection. On the other hand they tabulate the relatively high incidence of positive toxoplasmin skin tests in the normal population and show the significant rise in percentage of toxoplasmin-sensitive individuals which occurs with increase in age. Later, however, they recommend that serologic tests be performed in a reliable laboratory whenever possible. This is a judicious suggestion in view of their hypothesis that fetal pathology may occur as a result of placental transfer of toxic metabolic products of *Toxoplasma*. It would seem logical that an infection in the maternal organism which produced such substances would also be capable of eliciting an immunologic response which could be detected by serologic tests.

Of eighteen cases of embryopathy, three were diagnosed as toxoplasmosis. A complement-fixation titer of 1:80 and histopathological evidence of toxoplasmosis were shown for one infant who died postnatally. The other two infants were dead newborns in whom a diagnosis of toxoplasmosis was concluded on a basis of pathological findings.

These workers believe that maternal toxoplasmosis may affect the fetus in one or more of three ways (1) interference with maternal function, (2) production of toxic substances which pass through the placental barrier, and (3) direct invasion of the developing infant. Their evidence and discussion refutes the idea that a woman can

give birth to not more than one toxoplasmic infant.

Pyrimethamine was successfully used in the prophylaxis of habitual abortion and fetal and natal death. Perinatal mortality was reduced from 96% to 16%. However, these investigators wisely admit the possibility that the drug may be effective not only against *Toxoplasma* but also against other organisms. Furthermore, the drug may have some physiologic effect which is beneficial to pregnant women.

According to these investigators, toxoplasmosis should be suspected whenever there is habitual damage to and loss of fetus or newborn. They outline the procedure to be followed and give many suggestions which should be helpful to the obstetrician. Emphasis is placed on treatment with pyrimethamine; they suggest that more clinical trials are necessary before the drug can be recommended for routine use in suspected maternal toxoplasmosis. Obstetricians, pediatricians and others particularly concerned with *Toxoplasma* should find this article to be very interesting.

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Techniques in Tropical Pathology, by B. G. MAEGRAITH, Professor of Tropical Medicine, W. E. KERSHAW, V.R.D., Professor of Parasitology and Entomology, and D. DAGNALL, Chief Technician and Demonstrator in Parasitology, from the Liverpool School of Tropical Medicine. 164 + xii pages, illustrated. Edinburgh and London, Oliver and Boyd, 1961. 25s.

This first edition of a concise handbook is written "for the medical practitioner and laboratory technician working in small laboratories in the tropics." The book is divided into three sections; (a) use of apparatus, (b) examination of material and (c) diagnosis of specific conditions. An appendix, listing some laboratory equipment and normal values for blood and urine analyses is also attached. There are no references. The title of the book may be misleading to some American readers, since it pertains to clinical laboratory procedures.

The first section is primarily concerned with the use of optical equipment, especially compound microscopes. The description is concise and in order, but the usefulness of the section may be limited, since a diagram of a microscope showing parts and path of light rays is lacking.

The second section is subdivided into seven categories of materials examined in the laboratory. Useful laboratory procedures for the examination of specimens *i.e.*, blood, sputum, etc. are surveyed and described. Explicit and accurate descriptions are not always found for each. One might question whether some of the techniques outlined are best included in this section rather than in the following section. Greater consideration could be given to relative efficiency of the techniques discussed. There seems to be undue emphasis upon some of the complicated methods, such as electrophoresis of haemoglobins, whereas some important techniques, such as staining methods for intestinal protozoa, are omitted.

In the third section the authors give brief accounts of the life cycles of specific infectious agents and describe diagnostic features of the organisms involved. However, the descriptions are frequently too sketchy to be of much use to the non-specialist.

Errors, typographical and otherwise, are quite common. For example; opsonin is spelled "opsinin" (p. 46, l. 17); the reference to Ridley and Hawgood's Formol-ether method (p. 65, l. 20) has a confusing phrase, "8–10 ml. per cent formol-saline"; conjunctiva is spelled "conjunctive" (p. 108, l. 24); *Bulinus* (syn. *Physopsis*) (p. 137, l. 26) is used as a synonym of *Biomphalaria*; and the explanation for Fig. 7, Plate III is missing.

Material presented in this volume is covered in more detail in the available texts of clinical laboratory diagnosis and tropical medicine, but there may be some advantage in making it available in one small handbook.

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Toward the Conquest of Beriberi, by ROBERT R. WILLIAMS. 338 + xxii pages, illustrated. Cambridge, Harvard University Press, 1961. $7.50.

This is the story of thiamine—its relation to beriberi, its structural identification, its synthesis, its metabolic functions and its enrichment of cereals—told by the man who had most to do with its elucidation. The story is largely autobiographical. Young Williams, son of missionaries to India, and a recent graduate in chemistry from the University of Chicago, joined the staff of the Bureau of Science in Manila in 1909. The following year, Edward B. Vedder, Medical Corps, U. S. Army, brought him an alcoholic extract of rice polishings and asked him to find out what constituent of it prevented polyneuritis in chickens, which he believed was the same disease as human beriberi. This was the beginning of nearly half a century of research and campaigning, most of it as an avocation.

The author first reviews the history of clinical

beriberi, in western countries mainly on long sea voyages, in the Orient mostly in countries where polished rice was the staple food. He reviews the early theories of the cause of beriberi—toxin, infection and deficiency, and combinations of these. Then follow the experimental work on polyneuritis in chickens, initiated by Eijkman in Java and confirmed by workers in Japan, Malaya, the Philippines and elsewhere, and the establishment of its close relationship to the human disease. Much information and many quotations are taken from the classic monograph "Beriberi" published by Vedder in 1913.

Two chapters are devoted to the pathology and symptomatology of adult and infant beriberi, after which the preliminary work with Vedder is reviewed. This consisted mainly of the diffusion of the active substance through a parchment membrane, precipitation by phosphotungstic acid, absorption on bone char, and attempts to improve the yield of the substance by other methods.

On return to the United States in 1915, Williams joined the Bureau of Chemistry of the Food and Drug Administration, and continued his work in Washington. In 1919 he became Chemist at the Bell Telephone Laboratory in New York to work on submarine cable insulation. He was allowed to continue his vitamin research as an avocation. He obtained small grants, employed assistants, and worked with them evenings and week-ends. Other workers, including Seidell in Washington, Jansen and Donath in Java, Windaus in Germany and Ohdake in Japan, also made important contributions toward the isolation of the vitamin in crystalline form and the calculation of its empirical formula. Williams and his group accomplished this in 1933, and then went on to the fractionation of the molecule, the identification of the pyrimidine and thiazole fractions in 1935 and the synthesis of the vitamin in 1936. The author gives full credit to others who also accomplished the fractionation and synthesis by different methods. Soon more efficient and cheaper methods of synthesis were developed.

The author here interrupts the chronological story to acquaint his non-chemical readers with enzymes, coenzymes and the part that thiamine pyrophosphate has been found to play in carbohydrate metabolism. Beside its coenzyme function in the tricarboxylic acid cycle he lists 23 other enzyme systems in which it functions. He also discusses other phases of the chemistry of thiamine, including inactive derivatives, antivitamins and thiaminases. Then follow sections on thiamine in foods and tissues, the human requirement for thiamine, and thiamine in breast milk in relation to infantile beriberi.

Resuming the chronological story, the author next introduces a defense of his actions in connection with patents of the procedures in the synthesis of thiamine, which have been the subject of criticism. With no desire for profit, and in order to prevent the premature granting of United States patents to the I. G. Farbengesellschaft, Williams successfully applied for such patents, and assigned them to Research Corporation in New York, whose income is all spent in the support of academic research. After reserving modest royalties for him and his co-workers as their principal source of income, Research Corporation established the Williams-Waterman fund, which received fifty percent of all later royalties for the purpose of supporting nutrition research. To date the royalties have brought Research Corporation nearly seven million dollars, and the Williams-Waterman Fund four and one half million dollars.

The author then relates the licensing of the manufacture of thiamine to two Pharmaceutical firms and the tremendous increase in its production to 100 metric tons a year, with reduction in the price to four cents a gram. The first practical application of commercial manufacture was in the United States, where the Food and Nutrition Board established standards for enriched bread which were adopted by the Food and Drug Administration. After a campaign throughout the country led by Williams, millers finally agreed to market only enriched white flour. Later corn and rice enrichment were encouraged by laws in some southern states.

After World War II the author returned to the Philippines where, with the cooperation of Dr. Juan Salcedo, Jr., he conducted the Bataan experiment, adding enriched rice kernels (premix) to polished rice in certain towns on the Bataan Peninsula, using other towns as controls and basing results on the occurrence of clinical cases and deaths from beriberi. The value of the procedure was amply demonstrated, and in 1952 a rice enrichment law was passed by the Philippine Legislature. Unfortunately administrative difficulties have prevented the application of the law, and voluntary enrichment has been minimal.

The author records his observations of the present status of beriberi in individual countries of the Orient and Latin America as the result of a recent tour. Then follows a chapter on needed further research and a final one entitled "How to eradicate beriberi", in which he endorses the recent "Fifth Report of the Joint FAO/WHO-Expert Committee on Nutrition" and adds modifications and suggestions from his own experience.

This book will undoubtedly become a classic, as a joint biography of beriberi and Robert R.

Williams. The foreword by W. H. Sebrell adds an appreciation of the author. The references, about 900 in number, fully document the text. The portraits of the author, Dr. Salcedo and six of the pioneers are valuable additions. The spirit motivating the author is revealed by the last four lines of his two-verse sonnet "Chemistry of the Spirit" reprinted in the book:

"Can you but stimulate the hormones of high hope
Suppress those of despair and hate, kindle a sense of beauty,
Picture the glories of a growing race, the destiny of man,
You have a part in the creation; you too are very God."

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NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene—the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which entails a certain delay in their appearance because of the contractual limitation on the number of journal pages available for each volume. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the Journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the Editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

1. *Manuscripts* should be typewritten in *English* on *one side* only of the paper and submitted *in duplicate*. *Double spacing* should be used throughout the manuscript including the list of references, footnotes, and quotations.

2. The author should supply, typed on separate sheets, (a) a short title not over 50 letters for use as a running head; (b) the full title; (c) the authors' names and affiliations; (d) tabular matter; (e) footnotes; (f) references; (g) legends for illustrations; and (h) displayed formulas occupying more than 1 line of type.

3. *Tables* should be typed on separate sheets and be serially numbered in Arabic numerals.

4. *Illustrations* should be protected with cardboard to avoid creasing in the mails; illustrations larger than 8½ x 11 inches should be accompanied by smaller photographs. Only glossy prints can be used. Line drawings and graphs should be made with India ink and submitted on white or blue tracing paper; they should be legible after reduction to one column. Colored illustrations can be reproduced only at the expense of the author.

5. *Legends* should be separate and not attached to, or written on, the illustration copy.

6. *Positions for tables and figures* in the text may be indicated on the margin of the text pages.

7. All papers should end with a brief *summary*.

8. *References* should be cited by consecutive numbers in the text, and so numbered and listed in the list of references at the end of the article, without regard to alphabetical arrangement. References should be written as follows:

> 1. Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**: 493–497.

9. *Proprietary names* of drugs or chemicals registered by commercial pharmaceutical firms may not be used in the text if there is a generic name available in the U. S. Pharmacopoeia, New and Nonofficial Remedies of the A. M. A., or standard medical dictionaries. In certain cases where the generic name is relatively unknown, the registered name may follow it in parentheses in the title, at the first citation in the text, and in the summary.

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